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EXPERIMENTAL STUDIES ON DENGUE

I. ISOLATION, IDENTIFICATION AND MODIFICATION OF THE VIRUS

SUSUMU HOTTA

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In August, 1942 an epidemic of dengue broke out suddenly in the Nagasaki-Sasebo area of Japan and then in the Osaka-Kobe district. The infections originated from seamen of the Southern Line who fell ill on the homeward voyage and brought the disease into Japan proper. The epidemic ceased in November, but the next summer it started up again and recurred every summer until 1945. Meanwhile, other cities (Hiroshima, Kure and Fukuoka) were also infected. These cities are all situated on the seacoast between 33 and 35 degree north latitudes. The number of typical cases was estimated at 200,000, but the total number including abortive or suspected cases is considered to be much greater. The author has conducted observations and experiments on the disease since the first outbreak in Nagasaki.

Isolation of the dengue virus by the mouse brain passage method

White mice weighing 6 to 7 g were used. The original inoculum was heparinized or defibrinated blood taken from dengue patients within 48 hours after the first rise of temperature. With these materials the brain passage experiments were carried out by injecting 0.02 ml of a 10% Tyrode-diluted brain emulsion into mice of the next generation intracerebrally. Three strains were isolated, as shown in table 1. (See also fig. 1.)

TABLE 1.—*Dengue virus strains isolated.*

Name of strain	Time of isolation	Place of isolation	Number of generations until May, 1951
Mochizuki	Sept. 1943	Nagasaki	124
Sota	Sept. 1944	Osaka	76
Kin-A	Aug. 1944	Kyoto	70

Symptoms observed in the infected

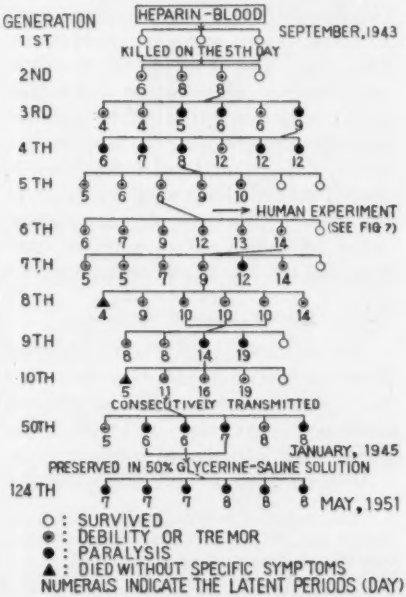

FIG. 1.—Transmission of dengue virus into brains of white mice. Death occurred usually within 24 hours after the appearance of symptoms.

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The research was conducted under the National Research Council of Japan (1942–1946).

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The author wishes to express his gratitude to Dr. Ren Kimura, director of the Institute, and to Dr. Albert B. Sabin, The Children's Hospital Research Foundation, Cincinnati, for helpful advice in the experimentation and in the preparation of the manuscripts.

mice were debility, tremor or paralysis. The paralysis was mostly of flaccid type and seen mainly in the hind legs. Incubation period and lethal rate were somewhat inconstant during the earlier generations, but after a number of passages they became fixed. The intracerebral LD_{50} titer was in the range of 10^{-5} to 10^{-6} after the 10th generation in the mice mentioned above. The virus could also be demonstrated in the blood of mice infected by the intracerebral route.

The gross changes in these mice were congestion of brain and lungs. In some cases small petechiae were seen on the meninges or on the surface of the lungs. In other organs, generally, a hemorrhagic tendency was observed, although after a number of passages its degree decreased. Histologically, a notable picture was found in the nervous system. Cell infiltration and glial proliferation were seen, and the neurons exhibited degenerative changes of varying degree. In the lungs congestion of the capillary vessels, corresponding to the macroscopic findings, and cell infiltrations in the interstitial tissue were observed. In the other organs, generally, the walls of arterioles and venules were affected. Figures 2 through 6 show these changes.*

Identification of the virus

Histopathology, incubation in bacterial mediums and filtration tests with brain emulsions from mice so infected confirmed that the infective agent transmitted into the mice was a virus.

The virus was nonpathogenic for white rats, rabbits or guinea pigs through various routes of inoculation. This particular host range and the biological features observed in the infected mice suggested that the virus was different from that of Theiler (1934) or of Traub (1935) and that of the Lansing type poliomyelitis. Analogously, the virus differs from the Coxsackie virus which was recently discovered (Dalldorf and Sickles, 1948). The virus was distinguished immunologically from Japanese B encephalitis virus.

Healthy adults showed the typical dengue symptoms after an intracutaneous injection of brain emulsion from infected mice, under the condition that possibilities of natural infection of dengue were strictly eliminated. One example is shown in figure 7. The individuals so infected were refractory when, 2 to 7 weeks after the disappearance of the symptoms, they were inoculated with the virulent dengue virus of human origin.

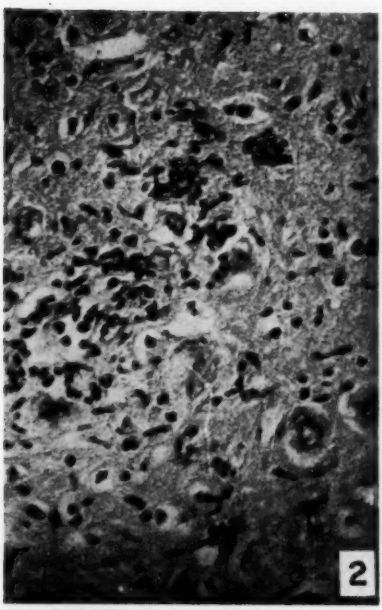
Convalescent serums of dengue patients, infected either experimentally or naturally, neutralized the isolated virus in vitro, while the control serums, those of persons with no previous history of the disease, did not.† The technique of testing was the same as with other viruses. The virus-serum mixtures were kept at 37 C for two hours, at 4 C for two more hours and then injected into mice intracerebrally.

Based on the results above, the Mochizuki strain of virus was identified

* The author is indebted to Dr. Shigeyasu Amano and Dr. Yoshiho Hashimoto of Kyoto University for these preparations.

† At least within the scope of the author's observations, no normal neutralizing antibody against this virus has yet been found in the people of Japan.

FIG. 2.—Brain, grey matter. Cell infiltration and glial proliferation. Hematoxylin-eosin stain. ×448.
FIG. 3.—Spinal cord, lumbar region. Cell infiltration. Hematoxylin-eosin stain. ×70.
FIG. 4.—Brain. Necrosis in cortex. Nissl stain. ×62.
FIG. 5.—Higher magnification of figure 4. Degeneration of the neurons. ×620.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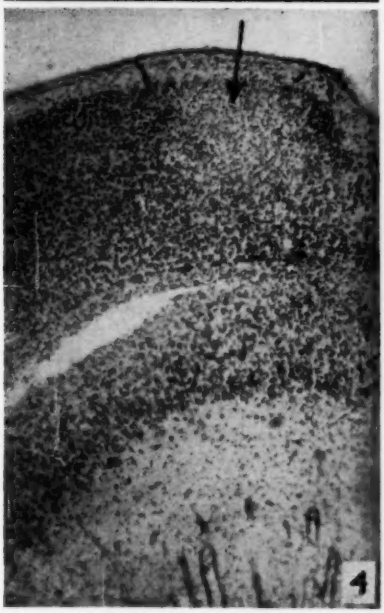
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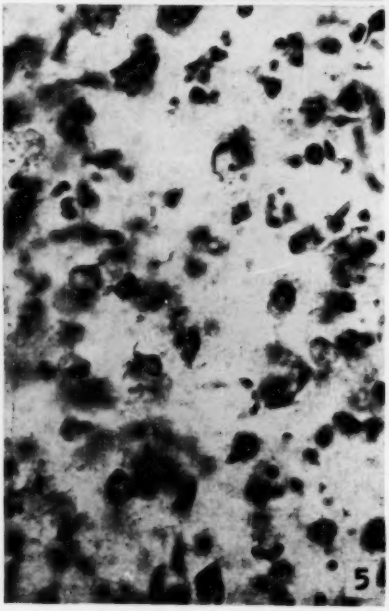
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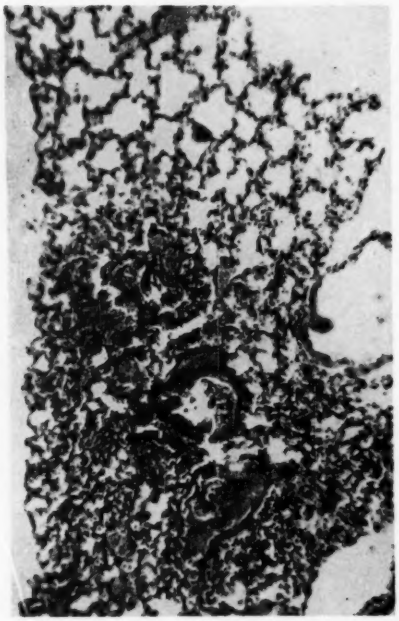

FIG. 6.—Lung. Capillary congestion and cell infiltration in the interstitial tissue. Hematoxylin-eosin stain. ×78.

as dengue virus, although 10 experiments on mosquito transmission (Sabin, 1948a) have yet been conducted. The other two virus strains, Sota and Kin-A, could not be distinguished from the Mochizuki strain virus either biologically or immunologically. It was concluded from these facts that these three strains of virus were certainly the virus of dengue.

Modification of the virus by mouse brain passage

In the course of consecutive transmission from mouse to mouse, alteration appeared in the properties of the dengue virus, changes being recognized in its pathogenicity for human beings or in findings from affected mice.

Pathogenicity of the virus for human beings.—As far as an intracutaneous injection of 0.2 ml of 10% mouse brain emulsion is concerned, pathogenicity of the Mochizuki strain of virus for human beings diminished gradually and finally was lost. The aspects are summarized in table 2.

Findings in affected mice.—(a) The incubation period and lethal rate of the murine infection were somewhat inconstant during earlier generations, but

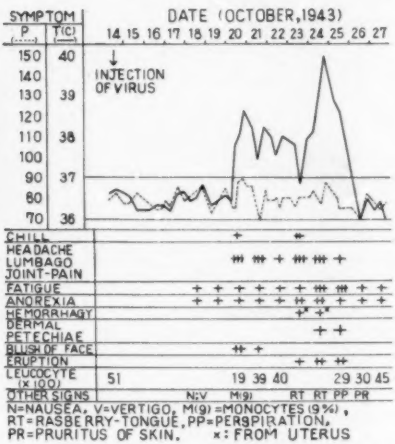

FIG. 7.—Progress of experimental dengue infection in man. The 5th generation of the mouse-transmitted Mochizuki strain was used, and 0.2 ml of 10% brain emulsion was injected intracutaneously.

after a number of passages the latent period was fixed and the lethal rate was practically 100%. (b) In the earlier generations more mice showed only debility or tremor but not paralysis, while in later generations the majority of mice showed paralysis. (c) During the earlier generations the hemorrhagic change was found in a majority of the dead mice, but after a number of passages a smaller percentage of mice showed the change. These aspects of the Mochizuki strain, which were very

TABLE 2.—*Changes in the human pathogenicity of dengue virus in the course of mouse brain passage (Mochizuki strain).*

Volunteer		Number of mouse passages	Time of inoculation	Incubation period (days)	Symptom							Degree of human-pathogenicity†
					Fever		Headache, lumbago and joint pain	Fatigue and anorexia	Blush of face and skin eruption	Hemorrhagy or dermal petechiae	Leucocyte* count (×100)	
Sex	Age				Duration (days)	Maximum (C)						
F	55	5	Oct. 1943	5	6	40.0	+++	+++	++	+	19/51	+++
F	31	16	Feb. 1944	6	5	39.6	++	++	+	+	28/59	++
M	23	16	Feb. 1944	6	1	37.0	+	+	−	−	45/60	+
M	31	17	Mar. 1944				−	−	−	−	47/55	−
M	25	33	June 1944	2	3	37.5	+	+	−	−	38/59	+
M	25	35	July 1944				−	−	−	−	53/54	−
F	47	35	July 1944				−	−	−	−	51/57	−
M	21	37	July 1944				−	−	−	−	52/58	−
M	25	37	July 1944				−	−	−	−	49/55	−
F	17	40	Aug. 1944				−	−	−	−	54/57	−
F	26	40	Aug. 1944				−	−	−	−	53/52	−
M	27	40	Aug. 1944				−	−	−	−	50/56	−
M	27	60	July 1945				−	−	−	−	55/58	−

* Numerator = minimal number during examination period; denominator = number before the infection.
† Measurements of the mouse-LD_{50} indicated that the amount of virus contained in the inoculum was almost equal in each instance.

similar to the Sota and Kin-A strains, are summarized in table 3 and indicate a concurrent relation in the shifts of the elements above as well as of human pathogenicity.

The virus was neutralized specifically by dengue convalescent serum, even after it had changed. No evidence has been found, at least from immunological tests, that another mouse neurotropic virus takes the place.

The conclusion was drawn from these results that the dengue virus changed its properties during the course of mouse brain passage, wherein especially it lost its original human pathogenicity.

Immunogenicity of the modified virus for human beings

The question of the immunogenic properties of the modified virus was investigated as follows.

Protection from a challenge with unmodified dengue virus.—Volunteers were injected intracutaneously with 0.2 ml at first, and then after a week with 0.3 ml of active modified virus suspension (Mochizuki strain) which had been

TABLE 3.—*Shifts of the properties of dengue virus in the course of mouse brain passage (Mochizuki strain).*

Virus		Mice				
Generation	Pathogenicity in human beings*	Number of mice observed	Incubation period (days)†	Lethal rate (%)	S1/S2‡	Percent of cases in which the hemorrhagic changes were observed
2– 5	+++	38	8.4±1.1†	86.3	2.38	75.0
6– 10		94	10.9±1.0	83.0	3.26	92.9
11– 15		140	11.3±0.5	64.3	2.65	90.8
16– 20	++, +, −	128	9.5±1.0	85.9	1.03	69.8
21– 25		52	10.4±0.3	88.5	0.86	57.8
26– 30		37	8.7±0.5	100.0	1.35	44.0
31– 35	+, −, −	48	9.1±0.6	89.6	1.13	33.4
36– 50	−, −, −, −, −, −, −, −, −, −	91	8.4±0.3	95.6	0.57	32.9
101–108		59	7.7±0.4	100.0	0.11	19.8

* Each notation represents one case.
† Calculated under the degree of confidence of 95%, applying the F-distribution-table. Ex: 8.4–1.1 and 8.4+1.1 are confidence limits (8.4 is the sample mean). Death usually occurred within 24 hours after the distinct appearance of symptoms. Mice which died within 24 hours after inoculation were rejected from the observation.
‡ S1 = number of mice showing only type I symptoms (debility and tremor); S2 = number of mice showing type II symptom (paralysis) with or without type I symptoms.

TABLE 4.—*Experiments of human immunization with the active modified dengue virus.*

Volunteers			Production of neutralizing antibody	Infection*
Group	Sex	Age		
Immunized	M	21	+	±†
	M	25	+	−
Control	M	34	−	+++
Immunized	F	17	+	−
	F	26	+	+
	M	27	+	−
Control	F	44	−	+++

The experiments were conducted during the period from July to Sept. of 1944. No possibilities of natural infection of dengue were in evidence.

* Degree of the symptoms induced after the challenge, the symbols of which may be interpreted with those expressed in table 2.

† On the 10th day after the challenge there occurred a transient fever of 37.3 C. Except for that, no particular signs were found either subjectively or objectively. No conclusive evidence could be obtained about the cause of the fever.

obtained from brains of infected mice and adjusted to contain 10^3 times mouse-LD_{50} per 0.02 ml. No abnormal signs were observed either subjectively or objectively, except for slight pruritis or slight induration appearing at the injected site. Two weeks after the second inoculation the volunteers' serums were separated to test their neutralizing power in vitro against the mouse passage virus. Then the volunteers were injected intracutaneously with fresh serum from a dengue patient of the febrile stage. As a control, the same tests were made on other volunteers who had had no previous treatments. All investigations were carried on in a place where at the time there were neither typical nor suspected cases of dengue, and all volunteers were isolated in a hospital during the observation periods. The main points of these experiments are summarized in table 4. The immunized persons were protected from an attack with dengue virus virulent for nonimmunized individuals. Even when infection occurred in the immunized group, symptoms were milder than those of the control group. It was possible to conclude from these results that the modified virus was able to stimulate a solid immunity against the unmodified dengue virus in man. No experimentation has yet been conducted on protection from dengue challenged by infected mosquitoes.

Twelve adults living in the city of Osaka were injected intracutaneously first (Aug. 3, 1945) with 0.2 ml and next, after 8 days, with 0.5 ml of an equal ratio mixture of active virus suspensions prepared as above from the Mochizuki, Sota and Kin-A strains, respectively. All individuals showed no abnormal signs after the injections. They remained well during the Osaka epidemic of 1945, although the observations were not made under strict control.

Titration of neutralizing antibody produced after inoculation of active modified virus.—Four adults were inoculated with active modified virus suspension in the same manner as described for the protection tests above. Two subjects had a previous history of dengue, being infected 47 and 36 months ago, respectively, and the other two had none. They were all in good health for 6 months before and after the inoculations and had no chance of any

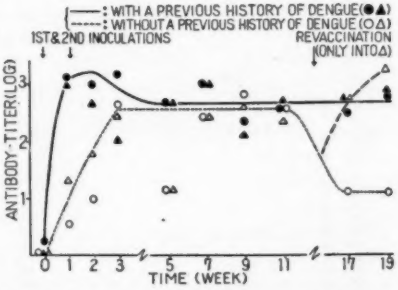

FIG. 8.—Shift of neutralizing antibody produced after inoculations of active modified dengue virus. Revaccination was made with an intracutaneous injection of 0.3 ml of the material 15 weeks after the 2nd inoculation.

vaccination. Their syphilitic seroreactions (Wassermann and Sachs-Georgi) were all negative. At various times the serum of each person was taken, and the neutralizing power to the mouse-fixed Mochizuki strain was measured. Technical principles of the titration were identical with those presented by Sabin (1948c), except that the dilutions of the elements were made with Tyrode's solution. Results obtained are illustrated in figure 8. (a) In the two normal persons the antibody titer reached its practical maximum 2 weeks after the second inoculation and remained at the maximal level for at least 8 weeks. (b) In the two subjects with previous history of dengue the antibody titer rose rapidly after the first inoculation, reached a maximum higher than that of those without history of the disease and was stable for 18 weeks, showing little fluctuation. (c) The titer increased promptly after reinoculation.

TABLE 5.—*Reisolation of the modified dengue virus from a volunteer injected with the active virus.*

Time of examination†	Blood		Milk	
	Number of mice‡	Incubation period (days)§	Number of mice	Incubation period (days)
0.0				
0.5	0/7			
1.0	0/9			
1.5	4/8*	9	3/5	12
2.0	3/8	9		
2.5	3/9	21		
3.0	2/7	9	3/5*	15
3.5	2/7	8		
4.0	0/5			
4.5	0/6			
5.0	0/5			
5.5	0/5		0/5	
6.0	0/5			
6.5	0/5			
7.0	2/5	7		
7.5	1/4	10		
8.0	0/4			
8.5	0/6			
9.0	1/6	8		
9.5	0/5		0/5	
10.0	1/5	7		
10.5	0/5		0/5	
11.0	0/6			
11.5	0/5		0/5	
12.0	0/5			
12.5	1/5	5		
13.5	0/5			
14.5	0/5			
15.5	0/5			

* Viruses which were unable to be distinguished from the original Mochizuki strain either biologically or immunologically were recovered by mouse brain passage.
† Number of days after the first injection.
‡ Numerator = mice showing the characteristic symptoms; denominator = mice inoculated with the materials, including the cases of noncharacteristic death.
§ The average, where two or more mice were involved.

Reisolation of the virus from a volunteer injected with active virus

A healthy adult was injected intracutaneously with a suspension of modified dengue virus (Mochizuki strain), the concentration of which was $10^{3.77}$ times mouse-LD_{50} per 0.02 ml. The injected dose was first 0.2 ml, and then 0.3 ml after 8 days. After the first injection, blood was taken every 12 hours and heparinized, and 0.02 ml of it was injected intracerebrally into mice weighing 6 to 7 g, to observe if it contained active virus. The same investigations were also made with milk because the volunteer was just in the lactation stage. The volunteer showed no abnormal signs either subjectively or objectively after the inoculations. Results obtained are shown in table 5.

From the mice marked with the asterisk in table 5, virus which could not be distinguished from the original Mochizuki strain, either biologically or immunologically, was recovered by mouse brain passage. One of the newly isolated strains was injected intracutaneously into a volunteer, but no abnormal signs were observed.

DISCUSSION

The work of Ashburn and Craig (1907) showed that the etiologic agent of dengue is a virus and that the virus is contained in the patient's blood during the febrile stage. Later Simmons et al (1931) reported on the experimental aspects of the disease. But many attempts to transmit the virus into ordinary laboratory animals and produce a specific change in them gave only negative results (Doerr, 1930; Sabin, 1948b). From 1943 to 1945, however, a number of Japanese investigators

(Kobayashi et al; Taniguchi et al, Yaoi and Arakawa, Ishii et al, Takenouchi et al, Toda and Nakagawa, Hayashi, Misao and Yamada), reported successful results, especially with mice, though not necessarily all of them received complete approval from other workers, and some were unable to prove their work with certainty (Hotta, 1947). During the same period a mouse-transmitted dengue virus was isolated by American investigators and comprehensive work of the subject was made with notable success (Sabin and Schlesinger, 1945; Sabin, 1948b). The author also had an opportunity to conduct research studies on the disease during the epidemic in Japan from 1942 to 1945 and was able to demonstrate three strains of the virus by injecting patient's blood into white mice intracerebrally. The mice inoculated with the virus showed definite symptoms characterized by debility, tremor or paralysis, and manifested marked pathological and anatomical changes, which included effects on the central nervous system and hemorrhagic tendency of varying degrees in different parts of the body. The virus was identified to be the virus of dengue, through procedures including human inoculation experiments and neutralization tests. Moreover, it has been reported that Sabin's virus and the author's, which were recovered independently, had the same biological and immunological properties (Sabin, 1948a and 1950). It can be established from these investigations that the isolation of dengue virus is possible, although fairly difficult, by the method of mouse brain passage.

Sabin and Schlesinger (1945) reported that their Hawaii strain of dengue virus was modified by propagation in mice. The author's studies conducted independently obtained results essentially identical with that of Sabin and Schlesinger: in the course of serial passage through mouse brains, human pathogenicity of the Mochizuki strain of dengue virus was gradually diminished and finally lost; but the individuals inoculated with the modified virus were refractory to subsequent injection of a highly virulent dengue serum, and virus-neutralizing antibody was shown to be produced in the serum. The shift of the neutralizing titers indicated that an anamnestic seroreaction was evidently present in the dengue antibody, too, and that a stronger immunity was acquired by repeated inoculations.

Sabin's studies (1948b) indicated that blood of persons inoculated with early passages of the mouse-adapted Hawaii strain of virus was able to cause a modified infection in human volunteers. Similarly, a modified infection was produced by the bite of mosquitoes which had fed on human beings inoculated with early passages of his mouse-passage virus. In the author's experiment a virus undistinguishable from the modified Mochizuki strain was isolated from the blood and also from the milk of a person injected with the active virus. The result suggested that the modified dengue virus multiplied in the human body without exerting a harmful influence upon the host or without recovering the original virulence. Further studies, however, are necessary to establish this conclusion which was made from a single positive result.

It has been established that vaccinia virus and fixed rabies virus are able to produce a solid immunity against smallpox and street rabies, respectively, in human beings if the inoculation occurs while the virus is active. Later, attenuated yellow fever virus was secured and is now in use for human vaccination (Theiler, 1948). Results obtained on dengue virus have been encouraging enough to conduct further investigations, including a field trial.

SUMMARY

During dengue epidemics in Japan from 1942 to 1945, three strains of dengue virus were isolated by injecting patient's blood, taken during the febrile stage, into brains of white mice and carrying on mouse brain passage thereafter.

Serial transmission of the virus through mouse brains resulted in a change in its properties. It lost its ability to induce in humans symptoms characteristic of the original disease, but was still able to produce in inoculated persons a protective immunity against virulent dengue virus as well as virus-neutralizing antibody.

Titration of the neutralizing antibody produced was performed. The virus was reisolated from a person so inoculated. The immunological and biological aspects have been discussed.

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EXPERIMENTAL STUDIES ON DENGUE

II. A SKIN REACTION OBSERVED DURING THE EPIDEMIC OF OSAKA, JAPAN, IN 1944

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From August to October, 1944 an epidemic of dengue occurred in the Osaka-Kobe district. The total number of typical cases was estimated to be at least 100,000. The location of the first onset was the Kitahama area, which includes a famous stock brokerage street with many banks and offices. The living standard of the inhabitants is relatively high. During the epidemic a skin reaction of the patients was observed and investigated.

MATERIALS AND METHODS

Twenty inhabitants of the infected area whose disease during the epidemic was diagnosed as typical dengue were examined. Eighteen of them were in the febrile stage, but two were tested after the disappearance of fever. No regard was paid to sex, age, occupation or living standard. Selection of the cases was made evenly within the infected zone, eliminating a regional partiality.

Antigen.—From mice showing typical signs of infection after an intracerebral inoculation of dengue virus, the brains were removed and ground into a homogeneous emulsion with Tyrode's solution. The emulsion was centrifuged at 3000 r.p.m. for 30 minutes, and the supernatant fluid was taken out. Its virus concentration was adjusted to 10^4 times mouse LD_{50} per 0.02 ml. The fluid was heated at 60 C for 60 minutes in a bath to inactivate the virus completely. The pH of the fluid was 7.4 to 7.6, owing to the buffer effect of Tyrode's solution. When the fluid was acid, it was adjusted to pH 7.4 by the addition of sodium hydroxide. If there was a sedimentation, centrifugation was repeated. No disinfecting agents such as carbolic acid or formalin were added, the entire procedure being carried out aseptically. The opalescent liquid thus obtained was used as the antigen and stored at 4 C.

One-tenth ml of the antigen above was injected into stretched skin of the patient's upper arm. Five to 10 hours after injection, induration appeared at the site and was accompanied by a red circular patch. In 5 cases vesicles were formed or necrosis appeared. Subjectively there were local pruritis and stinging. No cases of prolonged ulceration nor any with symptoms more severe or prolonged than those of the original process were found. The diameters of these reactions were measured at 24 hours and again 48 hours after injection.

Control test.—As controls, (a) fluid prepared from normal mouse brain in the same manner as the antigen was injected into the corresponding point of the opposite arm, and (b) the same observations were made after injecting the antigen and control fluids into healthy adults and into patients with other acute, febrile and eruptive diseases.

RESULTS

From the results obtained (tables 1 and 2) it was apparent that: (a) Dengue patients reacted specifically to the antigen. (b) Seven patients with scarlet fever showed no reaction; 5 with typhoid fever and one with murine typhus showed a distinct but generally lesser reaction. (c) Three healthy adults showed no reaction. (d) No group reacted to the control fluid. However, the reaction of the dengue group was not uniform. During the earlier stage of illness, on the first or second day of disease (8 cases), the reactions were somewhat weak and irregular 24 hours after injection and became more distinct after 48 hours. On the other hand, when antigen was injected on or after the third day of disease (12 cases), reactions after 24 hours were constant and somewhat more distinct than those after 48 hours.

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The author is indebted to Dr. Akimichi Inaba, Osaka, for the diagnosis of the patients, and to Dr. Yasuo Akizuki and Dr. Kotaro Okugawa of Kyoto University for the statistical analyses.

TABLE 1.—*Reactions of dengue patients.*

Day of illness	Fever above 37 C on day of examination	Antigen				Control fluid			
		Induration		Red patch		Induration		Red patch	
		24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.
		millimeters							
1	+	1.5	7.0	18.0	8.0	1.5	0.0	8.0	7.0
1	+	1.5	5.0	0.0	7.0	1.5	0.0	0.0	0.0
1	+	2.0	5.0	5.0	5.0	1.0	0.0	0.0	0.0
1	+	6.0	5.0	8.0	7.0	0.0	0.0	0.0	0.0
2	+	1.5	5.0	14.0	10.0	1.5	0.0	0.0	0.0
2	+	1.5	5.0*	14.0	8.0	2.0	0.0	8.0	7.0
2	+		5.0		8.0		0.0		0.0
2	+	5.0	7.0*	12.0	0.0	2.0	0.0	0.0	0.0
3	+	8.0	8.0	20.0	8.0	1.0	0.0	0.0	0.0
3	+		0.0		0.0		0.0		0.0
3	+	8.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0
4	+	5.0	2.0	8.0	5.0	0.0	0.0	0.0	0.0
4	+	10.0*	10.0*	12.0	12.0	0.0	0.0	0.0	0.0
5	+		5.0		5.0		0.0		0.0
5	+	7.0*	10.0*	8.0	12.0	2.0	1.5	0.0	0.0
6	+	7.0	2.0	8.0	5.0	0.0	0.0	0.0	0.0
6	+	7.0*	7.0*	10.0	10.0	2.0	1.5	0.0	0.0
6	+	7.0	8.0	8.0	12.0	0.0	0.0	0.0	0.0
6	−	6.0	3.0	8.0	0.0	0.0	0.0	0.0	0.0
7	+	7.0	7.0	8.0	8.0	2.0	1.0	0.0	0.0

* Vesicle formation or necrosis.

Statistical evaluation

Statistical analyses were made to clarify the results and to set a border line for positive and negative reactions. Samples of the dengue group (table 1) were divided as follows: (a) injected during the first two days of illness, induration, 24 hours; (b) injected during the first two days of illness, induration, 48 hours; (c) injected during the first two days of illness, red patch, 24 hours; (d) injected during the first two days of illness, red patch, 48 hours; (e) injected on or after the third day of illness, induration, 24 hours; (f) injected on or after the third day of illness, induration, 48 hours; (g) injected on or after the third day of illness, red patch, 24 hours; (h) injected on or after the third day of illness, red patch, 48 hours. Vesicle formation and necrosis were included in induration.

The mean of the population, which was assumed to be normally distributed, estimated under the degree of confidence of 95%, was calculated. Calculations were made using the small sampling method (Snedecor, 1940; Fisher, 1941), and the following values were obtained for groups (a) through (h), respectively:

TABLE 2.—*Reactions of control patients.*

Day of illness	Fever above 37 C on day of examination	Antigen				Control fluid			
		Induration		Red patch		Induration		Red patch	
		24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.
		millimeters							
Typhoid fever									
17	+	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0
20	+	5.0	5.0	8.0	8.0	0.0	0.0	0.0	0.0
21	+	4.0	4.0	5.0	5.0	0.0	0.0	0.0	0.0
26	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
42	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Murine typhus									
15	−	4.0	5.0	10.0	12.0	3.0	2.0	0.0	0.0
Scarlet fever									
5	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6	+	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	+	1.5	1.5	0.0	0.0	1.0	1.0	0.0	0.0
13	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	−	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

2.7 ± 1.8 mm, 5.5 ± 0.8, 8.9 ± 7.5, 6.6 ± 2.6, 7.2 ± 0.9, 5.2 ± 2.3, 10.0 ± 2.7, 6.4 ± 3.0. Values under the level of significance of 1% were rejected (according to Thompson, 1935), and each mean of universe of groups (b), (d), (e) and (g) was calculated again, obtaining values as follows: (b) 5.5 ± 0.8, (d) 7.6 ± 1.2, (e) 7.2 ± 0.9, (g) 8.9 ± 1.1. The means of universe of the typhoid fever group (table 2), calculated similarly, were as follows: induration, $+2.2+2.8$, red patch $\leqq 2.6+4.6$.

DISCUSSION

Antigen prepared from brain of mice infected with dengue virus was injected intracutaneously into dengue patients, patients suffering from other acute, febrile and eruptive diseases, and into healthy adults. Reactions from injection were an induration, a red circular patch or a combination of these two at the site, with vesicle formation or necrosis under certain conditions. Comparing the degree of the reactions (indicated by the diameter of the changes observed) of each subject, it was found that the reaction in dengue patients was constant and most distinct. However, the degree of reaction in dengue patients differed according to the stage of illness. The mean values obtained indicated that the reactions were practically constant 48 hours after injection of antigen for patients in the first two days of disease, and 24 hours for those injected on or after the third day of disease. The results seemed to show that the reactions were most distinct on the 4th, 5th or 6th day of disease or later in dengue.

Diagnosis of dengue is relatively easy during the epidemic except under the following conditions: (a) at the onset of the epidemic, (b) in the diagnosis of atypical cases and (c) in the differentiation from other acute, febrile and eruptive diseases found during the dengue epidemic. Isolation of the virus is usually difficult. Serological methods can be used. If it is shown that specific antibodies against dengue virus are absent or present in small amount in a patient's serum during the acute stage and have appeared or increased during the convalescent stage, the disease should be suspected of being dengue (Sabin, 1948; Schlesinger, 1949). The skin reaction described above may be useful as another method for diagnosis of dengue. Although the nature of this reaction has remained unexplained, it is rational to think that it is similar to that of lymphogranuloma venerum described by Frei (1925).

SUMMARY

Observations were made concerning a skin reaction after injecting dengue patients intracutaneously with antigen prepared from brains of mice infected with dengue virus. The reaction, appearing at the injected site as induration, red circular patch or a combination of these two, including vesicle formation or necrosis under certain conditions, was considered to be specific for dengue. The borderline of the positive appearance has been discussed statistically.

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A CLOUD CHAMBER FOR THE UNIFORM AIR-BORNE INOCULATION OF MICE

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It has been clearly pointed out by many investigators (see review by Rosebury, 1947) that the dynamics of air-borne infections can best be investigated by exposing experimental animals to atmospheres containing pathogens dispersed as fine droplets. Since extensive studies of respiratory infections with agents of the psittacosis-lymphogranuloma group were planned, a cloud chamber was constructed for the uniform air-borne inoculation of mice. Many features of cloud chambers already used at the University of Chicago in the Department of Medicine (Loosli et al, 1943; Sonkin, 1951) were included in the design. However, other features were added to provide for conditions of safety which would not limit research to agents nonpathogenic to man. The over-all design was kept as simple as possible, so that additional mechanical devices could be added as they were needed.

The present paper describes the cloud chamber and illustrates its operation with a bacterial and viral agent.

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MATERIALS AND METHODS

The mice used in these experiments were Swiss Albino (Webster strain) obtained from Markham Laboratories of Chicago, Illinois. On repeated examinations they were shown to be free from agents causing pneumonia in mice. Young mice weighing an average of 8 g as well as full grown ones weighing 25 g or more were used, but in any one experiment the mice were of the same age group.

A strain of *Serratia marcescens* producing an intensely red pigment was obtained from the departmental curator's collection. The strain of murine pneumonitis virus used in this investigation was first isolated by Gordon in 1941 and subsequently described by several investigators (Karr, 1943; Hilleman, 1945; St. John and Gordon, 1947; and Weiss, 1949).

Virus suspensions were prepared from heavily infected mouse lungs. Usually 5 or 6 lungs were placed in a tissue homogenizer and 12 ml of veal infusion broth added. The tissues were then homogenized, the suspension centrifuged for 10 minutes at 1200 r.p.m. to eliminate the larger tissue particles, and the supernatant quickly frozen and stored at −76 C. All virus pools were tested for bacterial contamination by inoculating thioglycollate broth tubes with 0.1 ml aliquots from each pool. Bacterially contaminated pools were discarded.

The statistical significance of differences in the mean survival time of the various groups of mice was determined by calculation of the standard error of the difference of the means, t and P, in the manner described by Mather (1947).

APPARATUS

The general arrangement of the laboratory apparatus is illustrated in figure 1. Briefly it can be described as follows:

The cloud chamber† (fig. 2) is of the dynamic flow type and is essentially a 7½ cubic foot aluminum box containing

† The cloud chamber was fabricated by Mr. Emil Mott, machinist of the Dept. of Anatomy of the University of Chicago.

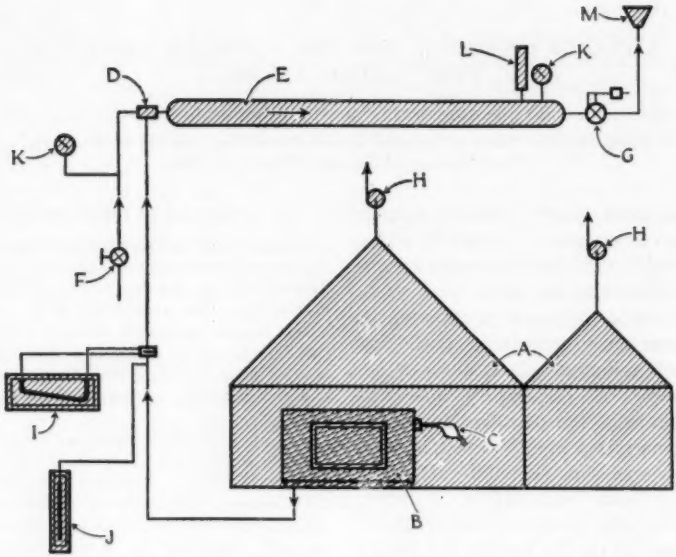

FIG. 1.—Arrangement of laboratory apparatus: A. Laboratory hoods; B. cloud chamber; C. atomizer; D. steam-jet evactor; E. retention chamber; F. steam control valve; G. back pressure control valve; H. hood exhaust fans; I. air flow meter (orifice type); J. manometer; K. pressure gauges; L. thermometer; M. exhaust head (above roof).

a single layer of cages capable of harboring 108 mice in individual compartments. The aerosol is produced by a modified DeVilbiss 40 atomizer (fig. 2 and 3) operated by 10 lbs. air pressure, and is rapidly mixed with air drawn into the chamber through 4 adjacent portholes. The aerosol-air mixture rapidly diffuses above and through the mouse cages and eventually flows into the exhaust pipe outlets. The atomizer and the exhaust outlets are located on opposite walls, the former above and the latter below the cages, in order to allow the most complete diffusion of aerosol-air mixture throughout the cloud chamber.

The flow system (fig. 1) serves the dual purpose of (1) diffusing the aerosol by drawing air at a continuous and uniform rate into and through the cloud chamber and (2) decontaminating the aerosol-air mixture after it leaves the cloud chamber. Air flow is produced by means of a steam jet evactor capable of drawing air at a maximum rate of 13 cubic feet per minute, or nearly two cloud chamber volumes per minute. The aerosol-air mixture leaving the cloud chamber is further drawn by the steam jet evactor into a retention chamber and maintained there at a temperature of 99 C, under 1 lb pressure, for a period of at least 30 seconds and finally eliminated through a back-pressure control valve to outdoors. Should the nature of the experiment require it, the flow system could be adapted to produce higher temperatures for decontamination, by increasing the steam pressure, modifying the steam-jet evactor design, and by increasing the pressure in the retention chamber.

The cloud chamber is enclosed in a

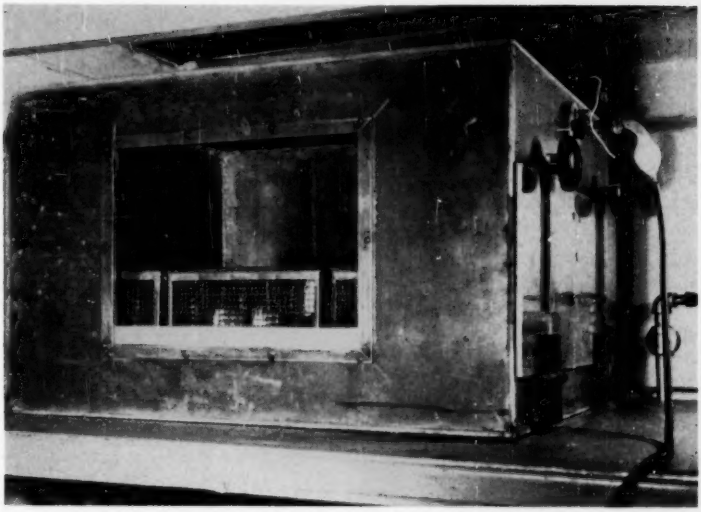

FIG. 2.—The cloud chamber.

double protective hood which has its own exhaust system and also ultraviolet lamps for decontamination.

The cloud chamber (fig. 2).—The cloud chamber is an aluminum box, made airtight by welding, 30 inches long, 24 inches wide, and 18 inches high. The front has a 16×12 inch leucite window, held firmly in place by metallic strips lined with rubber gaskets. It can easily be removed in order to clean the chamber between operations. The top has a round central window, 6 inches in diameter, which allows light to penetrate at an angle and illuminate the aerosol particles. The door is on the right side and is 10 inches high and wide enough to allow the tray supporting the mouse cages to be pulled in and out. It is well fitted with rubber gaskets and can be tightly closed. The portholes for the atomizer are located above the door in the center of the wall. The atomizer itself is fitted into a central 1 inch porthole of an air inlet diffuser plate having 4 additional adjustable ½ inch air intakes. Two ¾ inch exhaust air outlet pipes open on the other side below the cages, one near each corner, and are connected to the inlet of the steam-jet evactor.

The mouse cages (fig. 2).—An open metallic tray made of thin aluminum strips to allow free flow of air is inserted 4 inches above the floor of the cloud chamber. It supports 3 rows of 3 cages (9 in all) 9¼ inches long, 6¼ inches wide, and 3½ inches high. Each cage has 2 rows of 6 compartments, or 108 compartments altogether. The cages have sliding doors at the top and are entirely made of stainless steel wire mesh (3 meshes per inch) supported only by narrow metallic strips which do not interfere with the free flow of air through the cages.

The atomizer (fig. 2 and 3).—The atomizer used is similar to the De Vilbiss 40 type described by Rosebury (1947, p. 83). It operates on the principles that a jet of air, as it emerges from an orifice, induces a fall of pressure around it and aspirates fluid from an orifice placed in the low pressure area. Air is channeled through a straight capillary tube into the chamber containing the inoculum

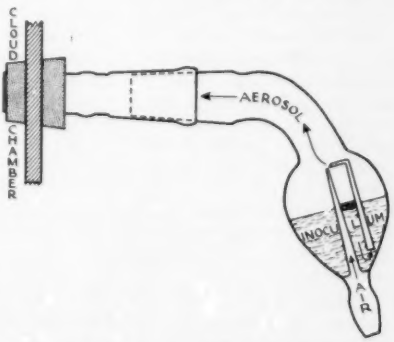

FIG. 3.—Diagram of the atomizer.

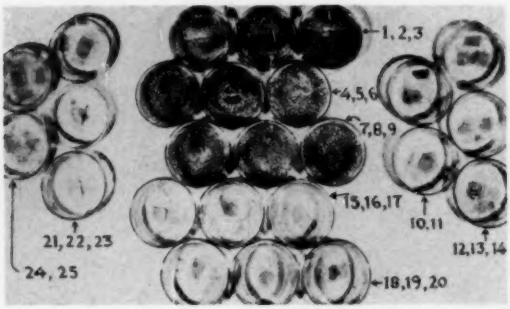

FIG. 4.— Nutrient agar plates exposed to an aerosol of *Serratia marcescens*. Plates 1–9 were placed inside the cloud chamber one on top of each cage; plates 10–25 were kept in various positions outside the cloud chamber to determine whether any organisms escaped from the cloud chamber or flow system under ordinary operating procedures. (See text for precise location.) Note the pigmented colonies covering plates 1–9 and completely absent from plates 10–25.

and opens approximately an inch above the level of the fluid. A fine spray is produced as the inoculum is aspirated from a contiguous capillary. The larger particles hit the walls of the chamber and fall into the main reservoir, and only the smaller ones escape through the opening, which is at a 105° angle to the flow of air. The atomizer differs from the standard De Vilbiss 40 in that a second opening near the orifices is absent and the outlet is fused to a half glass joint which may be inserted by means of a rubber stopper into the atomizer porthole of the cloud chamber. The capacity of the atomizer is about 10 ml and with a 10 lb pressure air line 8 ml are atomized in about 40 minutes.

The flow system (fig. 1).—The steam-jet evactor is an Elliott Company type "F", 1-inch size, operating at a maximum steam inlet pressure of 70 pounds per square inch (p.s.i.) and a retention chamber back-pressure of 1 p.s.i. gage. Air flow rate is measured by a Trimount model GE-4-3-E indicating flowmeter of the thin-plate orifice type having a maximum capacity of 13 cubic feet per minute (c.f.m.). A trimount model 2-12-3-E manometer indicates the degree of vacuum attained in the cloud chamber and exhaust piping and serves as a guide in the adjustment of the atomizer air inlet ports and steam-jet steam pressure. The retention chamber is of 8-inch steel pipe, 32 feet total length. A Boylston model 211, 2-inch, back-pressure valve regulates the pressure maintained in the retention chamber.*

The hood ventilation system.—To provide for additional safety for the operators, hood ventilation was calculated on the basis of an air velocity of 50 feet per minute across the face of the hoods with the doors wide open.

OPERATION OF THE CLOUD CHAMBER

Safety of operation.—Safety of operation of the cloud chamber is mainly based on the difference in air pressure between room and cloud chamber, maintained by the flow system. In ordinary runs the cloud chamber is evacuated at the rate of 1 to 2 cloud chamber volumes per minute. Because of the small size of the portholes and considerable airtightness of the chamber, this difference is sufficient to keep the aerosol well inside the chamber. The decontamination chamber keeps the exhaust air-vapor mixture for at least 30 seconds at 99 C and is believed to be sufficient to destroy nonsporeformers of ordinary heat resistance.

Additional protection in handling the animals after cloud chamber exposure is given by the negative pressure in the hood. Furthermore, under some circumstances the ultraviolet lamps may be used.

* Higher steam pressure would allow greater flexibility of operation. For example, with 150 p.s.i. of steam pressure 5 p.s.i. could be maintained in the retention chamber and the air in the cloud chamber exchanged at the rate of approximately 30 c.f.m.

The actual conditions of safety in the operation of the cloud chamber were tested in the following experiment (fig. 4): Nine open nutrient agar Petri dishes (1 to 9) were placed in the cloud chamber, one on top of each mouse cage. Other Petri dishes were placed outside the cloud chamber in various positions where aerosol might conceivably be expected to leak. Plates 10 and 11 were placed face down on the top window. Plates 12, 13, and 14 faced the atomizer, the air intakes, and the door at a distance of 4 to 6 inches. Plates 15, 16, and 17 faced the front window of the cloud chamber inside the hood, and plates 18, 19, and 20 were placed in a similar position just outside the hood. Plates 21, 22, 23 were placed at various places along the exhaust pipes. Finally plates 24 and 25 were placed on the roof facing the two exhaust outlets of the hood. No plates were placed near the cloud chamber steam exhaust, because the heat would have melted the agar.

A 24-hour veal infusion broth culture of *Serratia marcescens* was then atomized under conditions simulating a regular cloud chamber run. Before atomizing the broth culture, the steam was allowed to flow and bring the temperature of the retention chamber up to 99 C. Atomization was then started by means of a 10 lb pressure air line, while the air from the cloud chamber was evacuated at the rate of 1 volume per minute. After 40 minutes about 8 ml were dispersed and atomization discontinued. Air was allowed to flow through the cloud chamber for an additional 15 minutes and the door finally opened for 5 minutes. The plates were then covered and incubated for 24 hours at 37 C.

The results are shown in figure 4. The plates exposed to the aerosol in the cloud chamber were covered with innumerable typical red pigmented colonies. The plates exposed outside the cloud chamber contained variable numbers of common air contaminants. Plates 24 and 25 were especially heavily contaminated because air was funnelled through the exhaust systems of the hood onto them. However, red pigmented colonies were not found in any of the plates outside the cloud chamber.

The experiment clearly proved that atomized bacteria, under the conditions of the experiment, remained strictly confined to the cloud chamber and its exhaust system.

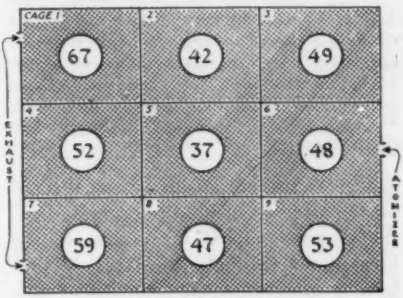

FIG. 5.—Distribution of bacteria throughout the cloud chamber. The numbers in each circle represent the colony counts of nutrient agar plates exposed to aerosols of *Serratia marcescens*, one on top of each cage.

Uniformity of distribution of the aerosol.—The principal purpose of the cloud chamber was to obtain a tool for the uniform air-borne inoculation of mice. The uniformity of distribution of the aerosol throughout the cloud chamber was tested in the following experiments:

Preliminary tests were performed with *Serratia marcescens*. As in the previous experiment, nutrient agar plates were placed on top of each cage and exposed to aerosols of diluted bacterial cultures. Figure 5 shows the added results of 3 runs. The number of organisms collected on top of each cage vary from 37 to 67 and differences do not appear significant.

The results of these preliminary experiments are only of limited value, be-

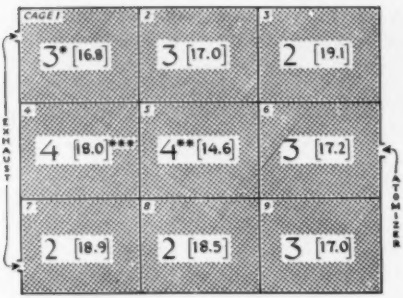

FIG. 6.—Effect of aerosol of murine pneumonitis virus inhaled by mice in the 9 cages of the cloud chamber. The large numbers represent mice dying in each cage after exactly 50% of the whole group had died. The numbers in brackets represent mean survival time in each cage. * Traumatic death; ** bacterial death; *** survival at 28 days.

cause (1) the organisms were collected on top and not inside the cages and (2) the plates modified the course of the flow of air through the cages. Therefore, a more accurate experiment was performed with the agent of murine pneumonitis by infecting 54 mice occupying alternate compartments in the cages.

About 8 ml of a 1:100 suspension of murine pneumonitis virus were atomized in about 40 minutes, while the air in the cloud chamber was exchanged at the rate of 13 cubic feet per minute. The position of each mouse in the cloud chamber was noted and its survival time recorded.

The results are summarized in figure 6. Note that two nonspecific deaths occurred (one traumatic and one bacterial), which are not counted. The mice died between the 12th and the 24th day, except for one which survived and was killed on the 28th day. The effect produced by the aerosol corresponded approximately to an intranasal inoculation of a 10^{-5} dilution of the same pool of virus. In figure 7 the distribution of deaths is recorded after exactly 50% of the mice had died. Obviously there is no significant difference among the 9 cages. This is even more apparent when the deaths occurring in 3 consecutive cages

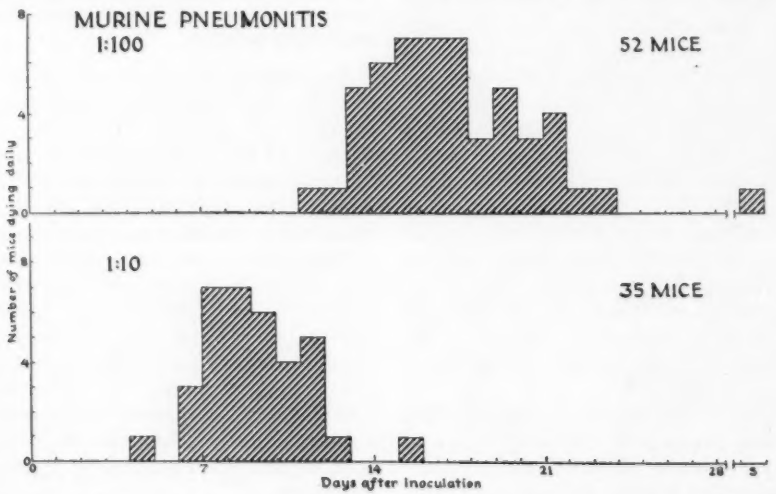

FIG. 7.—Distribution of mouse deaths after cloud chamber exposure to two concentrations of murine pneumonitis virus.

are added up. For example:

First row	3+3+2= 8	Total no. of mice, 17
Second row	4+4+3=11	Total no. of mice, 17
Third row	2+2+3= 7	Total no. of mice, 18

or

First column	3+4+2=9	Total no. of mice, 17
Second column	3+4+2=9	Total no. of mice, 17
Third column	2+3+3=8	Total no. of mice, 18

Figure 7 also records the average survival time of the mice in each cage. The only surviving mouse had extensive consolidation of the lungs when autopsied on the 28th day, and its survival time was recorded as 28 days. Differences in survival time among the various cages are remarkably small, with the exception of cage 5. The survival time of the mice in that cage is significantly lower than that of mice in other cages. For example, if the test for the significance of the difference of the means (t test) is applied, the probability (P) that the difference between survival time in cage 5 and the general average (17.5) is due to chance equals 0.02. On the other hand, the difference between the highest survival (cage 3, 19.1) and the general average is not significant ($P=0.22$). However, a concurrent unidentified bacterial infection possibly played a role in lowering the survival time in cage 5. In fact, one mouse in that cage had died of bacterial infection and others appeared sick during most of the latent period of murine pneumonitis infection.

In spite of this discrepancy, differences in the average survival times in any three consecutive cages appear insignificant:

First row	17.6 ± 0.6
Second row	16.6 ± 1.0
Third row	18.1 ± 0.8
First column	17.9 ± 0.8
Second column	16.7 ± 0.8
Third column	17.8 ± 0.6

The difference between the lowest of the above survival times (second row, 16.6) and the highest (third row, 18.1) has a P value of 0.15.

The results clearly indicate that under the conditions of the experiment mice are uniformly exposed to an aerosol in the cloud chamber.

AIR-BORNE INFECTION WITH THE AGENT OF MURINE PNEUMONITIS

The experiment just described indicates that inoculation of mice in the cloud chamber is very wasteful of virus. In fact, it is calculated on the basis of titration by the intranasal route that only 1/5000th of the virus actually reached the mouse lungs. Some of the loss can be prevented, without impairing the distribution of the aerosol or safety of operation, by evacuating the air in the cloud chamber at the rate of 6 cubic feet per minute instead of 13. Furthermore, the same inoculum can infect the full load of 108 mice as well as one only.

Humidity plays an important role in determining the effectiveness of inoculation. Most of the mice survive even with highly concentrated inoculums when the relative humidity of the room is 50% to 60%. These results agree with those reported by Rosebury (1947) for the agents of meningopneumonitis and psittacosis. Although the optimum humidity for the effective inoculation with the agent of murine pneumonitis has not been determined, excellent results were obtained when the relative humidity of the room was only 35%.

Figure 7 illustrates the distribution of mouse deaths after cloud chamber inoculation with 1:100 and 1:10 suspensions of murine pneumonitis virus. Two characteristics of the virus become apparent. The virulence for individual mice varies greatly. With the smaller inoculum, deaths extend from 12 to 24 days, with one survivor. With the more concentrated inoculum, deaths occur

between the 7th and the 13th day, with isolated deaths at 5 and 16 days. Furthermore, acquired resistance is gained very slowly by the mice, since mice continue to die for 24 days. In other experiments with even smaller inoculums deaths continue for as long as 40 days. Furthermore, mice autopsied at various intervals of time contain progressive lesions for 4 to 5 weeks.

Of interest also is the finding that mice of all age groups are highly susceptible to infection. Incomplete data even indicate that susceptibility increases with the advancing age of the mice.

SUMMARY AND CONCLUSIONS

A cloud chamber of the dynamic flow type for the simultaneous air-borne inoculation of as many as 108 mice has been constructed. The inoculation is effected by a modified De Vilbiss 40 atomizer operated at 10 lb air pressure. The aerosol is rapidly mixed with incoming air, the aerosol-air mixture diffuses above the cages, it is drawn through them, and eventually flows into the exhaust system. The exhaust is operated by a steam jet evactor which serves the dual goal of drawing air into and through the cloud chamber for the dual purpose of mixing the aerosol and pulling the aerosol-air mixture into the decontamination chamber, where it is maintained for at least 30 seconds at 99 C.

Extensive tests revealed that under ordinary operating conditions the inoculum is strictly maintained in the cloud chamber and its exhaust system. Furthermore, mice are uniformly infected throughout the cloud chamber. The rate of exchange of air through the cloud chamber (which may be varied from one to two volumes) and relative humidity play important roles in determining the effectiveness of inoculation with the agent of murine pneumonitis. It is believed that the cloud chamber thus developed is a valuable tool for quantitative studies of air-borne infections of mice.

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While this material was in press Mr. Frank M. Gogolak added important features to the cloud chamber which allow, among other things, careful control and measurement of the relative humidity of the incoming air and the particle size of the aerosol.

THE EFFECT OF THYROXINE AND RADIOACTIVE IODINE ON RESISTANCE OF MICE TO INFECTION WITH MURINE PNEUMONITIS VIRUS

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There is good experimental evidence for believing that the enzyme systems of virus-infected cells participate in the formation of new virus and that viruses multiply best in cells of high metabolic activity. This evidence has been obtained almost entirely from the study of bacterial viruses and of animal viruses growing in tissue cultures and chick embryos (for discussion, see Moulder and Weiss, 1951a). However, the more complex relationship between virus multiplication and the metabolic state of the intact animal host is by no means so clearly understood. While it is almost impossible to define the normal metabolic state of an animal, certain experimental procedures such as administering hormones or withholding vitamins may obviously produce alterations in the metabolic activities of the animal. The effect of several such procedures upon susceptibility to virus infection has been investigated. The relation of nutrition to experimental viral infection has been comprehensively reviewed by Clark (1949 and 1950). A variety of dietary deficiencies produced an increased resistance to virus infection, suggesting that an adequate supply of all essential metabolites favors virus multiplication. The relation to resistance to infection of several other factors which probably alter the metabolic state of the infected animal by raising or lowering the basal metabolic rate is more difficult to interpret. Pituitary growth hormone and testosterone enhanced the growth of influenza virus in mice, possibly by increasing the velocity of certain enzymic reactions concerned with protein synthesis (Kalter et al, 1950). Prolonged diethyl ether anesthesia increased the resistance of mice to several neurotropic viruses (Sulkin et al, 1946a and b and 1947). The ether concentration in the nervous tissue of the anesthetized mice was far below effective virucidal levels, and the authors concluded that a lowered metabolic rate in the infected cells probably accounted for the increased resistance. Acclimatization of mice to low environmental temperatures increased their resistance to poliomyelitis virus (Holtman, 1946a). Treatment of mice with a thyroactive iodocasein increased their resistance to infection with poliomyelitis virus, while treatment with thiouracil, which depresses thyroid activity, lowered it (Holtman, 1946b). However, Gollan (1948) found that the susceptibility of mice to infection with the MM virus of the encephalomyocarditis group was unaffected by various doses of crystalline thyroxine.

Although these regimens designed to alter the basal metabolic rate of the host produced conflicting changes in resistance to virus infection, the present investigation was undertaken in the belief that alteration of the basal meta-

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bolic rate of the infected animal is still the simplest and most direct way to test the assumption that viruses thrive best in the host with the highest rate of metabolism. The best way to change the metabolic rate appeared to be through raising and lowering the level of thyroid activity.* Therefore, mice were treated with thyroxine to increase their metabolic rate and with radioactive iodine I^{131} to destroy thyroid tissue and thus decrease their metabolic rate. The resistance of these mice to murine pneumonitis virus was then compared with that of untreated mice by infecting all three groups of animals by exposure to an aerosol of the virus in a cloud chamber. Under certain conditions, the mice given thyroxine died earlier than the controls, while the mice given radioactive iodine died later. These changes in susceptibility are in the directions predicted by the hypothesis that virus multiplication is favored by an active host cell metabolism. However, careful examination of the data indicates that such a simple unequivocal interpretation is unwarranted and that the exact role of the thyroid in resistance to infection with murine pneumonitis virus remains unknown.

MATERIALS AND METHODS

The strain of murine pneumonitis, th· ource of mice, and the method of infecting mice in the cloud chamber have been described by Weiss and Segeler (1952). All mice were kept on a complete dog food diet and water in a room maintained at 24 ± 2 C, except for experiment III, in which the temperature occasionally reached 33 C.

Crystalline thyroxine (Squibb) in 0.9% sodium chloride was prepared for injection by solution in the minimum amount of *N* sodium hydroxide and used the same day. Because tyrosine resembles thyroxine in chemical structure and solubility, tyrosine solutions were prepared in the same manner and injected into control mice in some experiments. Both solutions were injected subcutaneously in 0.1 ml amounts. After repeated injections, the mice sometimes developed zones of induration and necrosis, which promptly disappeared when the injections ceased. Except for a possible increase in respiration, the thyroxine-treated mice did not behave differently from untreated animals. Thirty uninfected mice were given thyroxine and none died as a result of the thyroxine administration.

Radioactive iodine I^{131} in the form of sodium iodide without carrier and dissolved in 0.9% sodium chloride was injected subcutaneously.† Each mouse received a single injection of either 0.08 or 0.10 mc radioactive iodine, an amount calculated to be sufficient for destruction of all the thyroid tissue (Gorbman, 1949). Mice which received radioactive iodine ate and drank less than untreated mice for a few weeks after injection, but otherwise resembled untreated mice of the same age group.

The oxygen consumption of intact mouse lungs was determined as in a previous paper (Moulder and Weiss, 1951b). The lower Q_{O_2} found here is probably a reflection of the older age of the mice. Liver slices were cut with the microtome described by Stadie and Riggs (1944) and their oxygen consumption measured under the same conditions used for lungs.

The statistical significance of the effect of thyroxine and radioactive iodine upon resistance to murine pneumonitis virus was determined by calculation of the mean survival time of treated and untreated groups of mice, the standard error of the difference of the means, t and P, in the manner described by Mather (1947).

EXPERIMENTAL RESULTS

The effect of thyroxine and radioactive iodine on thyroid histology and oxygen uptake of lung and liver.—Serial sections of the thyroid region of mice were taken to learn the effect of thyroxine and radioactive iodine on the thyroid gland. Thyroid tissue was readily found in each of ten mice from control groups. The thyroids of four mice which had received varying amounts of thyroxine were simi-

* While this paper was in press, the mechanism of action of the thyroid hormone was comprehensively reviewed by Barker (1951).

† Radioactive iodine was kindly supplied by Dr. Dwight E. Clark of the Dept. of Surgery, The University of Chicago. We are also greatly indebted to Dr. Walter E. Kisieleski of the Argonne National Laboratory for inoculating the mice with radioactive iodine.

TABLE 1.—*Oxygen consumption of livers and lungs from untreated, thyroxine-treated, and I^{131}-treated mice.*

Treatment	Organ	Q_{O_2}
None	Lung	5.6±0.2 (24)*
Thyroxine	Lung	6.0±0.2 (16)
I^{131}	Lung	5.8±0.3 (6)
None	Liver	6.7±0.3 (12)
Thyroxine	Liver	7.3±0.2 (5)
I^{131}	Liver	7.0±0.3 (6)

* Q_{O_2} (microliters oxygen consumed in 1 hour by 1 mg dry tissue) is given as mean ± S.E. (number of observations). These animals were taken from experiments I, II and IV.

lar to those of the control groups. Of six mice which received 0.08 mc radioactive iodine, no thyroid tissue could be detected in four and only a few small follicles were found in the other two. Four mice which received 0.1 mc radioactive iodine were examined, but no thyroid tissue could be detected in any of them.

On the basis of earlier studies (Gerard and McIntyre, 1933; Davis and Hastings, 1935; McEachern, 1935; McShan et al, 1947; Brophy and McEachern, 1949) it was expected that treatment of mice with thyroxine would increase the respiration of their isolated tissues, while radioactive iodine would lower it. However, table 1 indicates that treatment with neither thyroxine nor radioactive iodine had any significant effect on the Q_{O_2} of lungs and livers of the treated mice. The mice were given sufficient thyroxine to raise their basal metabolic rates (see Brody, 1934), and since the radioactive iodine effectively destroyed the thyroid tissue of the injected mice, those animals undoubtedly had subnormal metabolic rates. However, the oxygen consumption of their lungs, which is a good measure of metabolic rate of an organ or tissue, was not changed. Therefore, the alterations in susceptibility to infection with the virus of murine pneumonitis now to be described were probably caused by metabolic changes more complex than a quantitative shift in the level of metabolic activity in the infected cells.

The effect of thyroxine on resistance.—In each of three independent experiments mice treated with thyroxine died of murine pneumonitis faster than untreated controls. In experiment I, young mice 17 to 21 days old were injected with 0.1 mg thyroxine on alternate days. After seven injections, they were exposed, together with untreated mice of the same age, to an aerosol of murine pneumonitis virus of a concentration sufficient to kill untreated mice in about eight days. Thyroxine was also given on the first and third days after infection.

TABLE 2.—*Effect of thyroxine and radioactive iodine on the survival time of mice infected with murine pneumonitis virus.*

Exp. number	Group	Treatment				Number of mice			Mean Survival time ±S.E.	Significance of difference with group A, as P
		Substance given	Amount per injection	Number of injections: Before infection	Number of injections: After infection	Died	Survived	Total		
I	A	None				19	0	19	8.0±0.3	
	B	Tnyroxine	0.1 mg	7	2	22	0	22	5.7±0.3	0.001
II	A	Tyrosine	0.2 mg	4	4	14	0	14	9.8±0.4	
	B	Thyroxine	0.2 mg	4	4	14	0	14	6.9±0.3	0.001
III	A	None				14	1	15	17.3±1.1	
	B	Tyrosine	0.2 mg		10	9	3	12	19.7±1.8	0.23
	C	Thyroxine	0.2 mg		7	17	3	20	16.1±1.1	0.62
	D	Thyroxine	0.1 mg		10	17	1	18	13.0±0.5	0.002
	E	Thyroxine	0.2 mg		10	17	1	18	14.1±0.6	0.02
IV	A	None				16	0	16	7.6±0.3	
	B	I^{131}	0.08 mc	1		15	0	15	7.4±0.3	0.71
V	A	None				21	0	21	7.8±0.4	
	B	I^{131}	0.1 mc	1		22	1	23	11.0±0.9	0.003

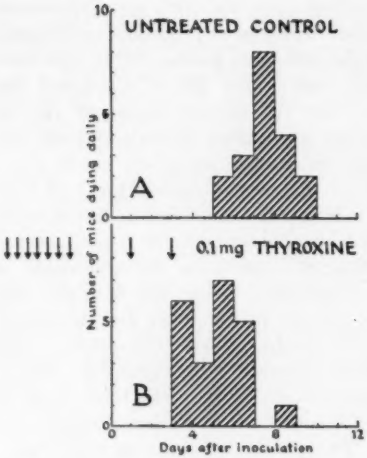

FIG. 1.—Effect of thyroxine on the survival time of mice infected with murine pneumonitis (exp. I).

As shown in table 2 (exp. I) and figure 1, the mice receiving thyroxine died much sooner than those receiving no treatment, despite the fact that the infecting inoculum killed all the animals in a relatively short time. The lungs of the thyroxine-treated mice while in some cases not as consolidated as those of control mice, contained many elementary bodies, and the treated mice probably died of murine pneumonitis.

Experiment II was performed in a similar fashion with adult mice 11 weeks old and weighing about 25 g. One group received eight injections of 0.2 mg thyroxine, four before and four after infection in the cloud chamber. The control group was similarly injected with 0.2 mg tyrosine. Here also the mice receiving thyroxine died significantly earlier than the controls (table 2, exp. II; fig. 2). The lungs of the thyroxine-treated mice contained extensive lesions but were less consolidated than the lungs of the tyrosine-treated mice. There was some indication that the mice injected with tyrosine lived about one day longer than untreated animals, but the experiment was not designed to show this difference clearly.

Experiments I and II clearly demonstrated that thyroxine administration hastens the death of mice infected with murine pneumonitis virus, but they also posed some new questions. Do the thyroxine-treated mice die sooner than untreated animals because they inhale a larger inoculum of virus due to their increased rate of respiration? Does thyroxine act, not by increasing the growth rate of the virus, but by rendering the treated mice more susceptible to the lethal action of the virus? Do varying amounts of thyroxine have different effects on the survival time of mice? And finally, does tyrosine act in an opposite manner to thyroxine by prolonging the life of mice with murine pneumonitis?

Experiment III was planned to answer these questions. The general plan of the experiment may be gathered from figure 3 and table 2. A large group of previously untreated mice was infected in the cloud chamber with only about one-fifth the inoculum of virus given in previous experiments to produce a long

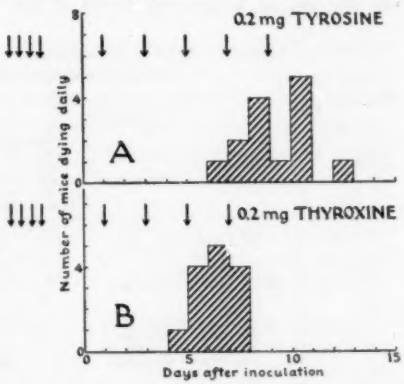

FIG. 2.—Effect of thyroxine on the survival time of mice infected with murine pneumonitis (exp. II).

incubation period in which thyroxine and tyrosine might be allowed to exert their effects after infection with the virus. The mice were then divided at random into five groups (table 2). The mice of group A received no treatment and served as untreated controls. Group B contained mice which received 0.2 mg tyrosine on alternate days after infection. The mice of group C were injected with 0.2 mg thyroxine on alternate days starting with the seventh day after infection. The mice of groups D and E received 0.1 and 0.2 mg thyroxine, respectively, on alternate days starting with the first day after infection. In addition, ten untreated mice as in group A and ten treated with 0.2 mg thyroxine as in group E were killed, five on the seventh and five on the ninth day after infection, and pools were prepared from their lungs for determination of virus titer.

A few mice survived the lower infecting dose of experiment III and complicated calculation of the mean survival times. Of the 83 mice infected, 11 were still alive on the 28th day when they were killed and autopsied. One mouse in group A and one in C had lost weight, had almost completely consolidated lungs, and probably would have died within a short time. They were arbitrarily recorded as having died on the 30th day after infection. The remaining nine mice had gained weight, had only minor pulmonary lesions, and were obviously recovering. They were called survivors and were excluded from calculation of mean survival times. Two mice in each of groups D and E died on the eighth day and were discarded because it was not known whether or not they died of murine pneumonitis.

The results of experiment III, shown in figure 3 and tables 2 and 3, suggest the following conclusions:

a. The multiplication of murine pneumonitis virus is neither increased nor decreased by treatment of infected mice by thyroxine (table 3). The concentration of virus in the lungs of mice from groups A and E was approximately equal at both seven and nine days after infection. It is of interest that the virus titer apparently begins to decline several days before most of the mice die.

b. The effect of thyroxine in decreasing the survival time of mice infected with murine pneumonitis virus is not due to inhalation of a larger infecting dose by the thyroxine-treated animals. The mice of groups D and E died faster than untreated or tyrosine-treated controls, although thyroxine was not administered until after infection had occurred.

c. Thyroxine does not act by making the mice more susceptible to the terminal lethal effects of the virus, because the mice of group C, which were given thyroxine late in the course of infection, after the peak virus titer had already been reached, did not die significantly faster than the untreated mice of group A. However, it is possible that mice treated with thyroxine throughout the entire course of infection finally become more susceptible to the cumulative lethal effect of virus growth.

d. There was no significant difference in the survival times of mice given 0.1 and 0.2 mg thyroxine. For the significance of the difference in mean survival time between groups D and E, $P = 0.1$–0.2. Therefore, it is probable that even larger amounts of thyroxine would not cause a further marked increase in the susceptibility of mice to murine pneumonitis virus.

e. Although tyrosine did increase the mean survival time of mice, the effect was not significant ($P = 0.23$). However, if χ^2 is calculated from a fourfold table constructed on the basis of the number of mice in groups A and B alive or dead on the 17th day, the corresponding

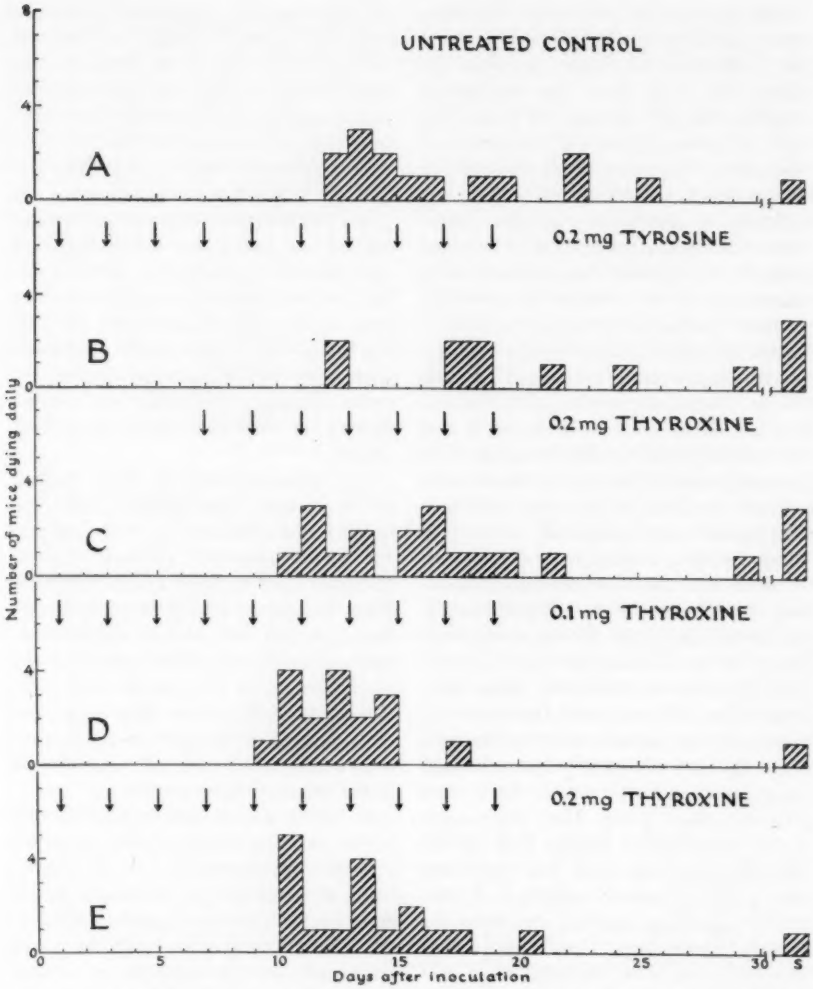

FIG. 3.—Effect of thyroxine and of tyrosine on the survival time of mice infected with murine pneumonitis (exp. III).

value of P is 0.06, just on the borderline of significance. Thus, under more favorable conditions, tyrosine may be found by some unknown mechanism to significantly increase the resistance of mice to murine pneumonitis.

The effect of radioactive iodine on resistance.—Two independent experiments were carried out on the effect of radioactive iodine on resistance to murine pneumonitis virus. In experiment IV, a single intravenous injection of 0.08 mc radioactive iodine in 0.4 ml 0.9% sodium chloride was given to mice three weeks old and about 10 g in weight. Three months later the treated mice, to-

TABLE 3.—*Effect of thyroxine on virus titer in the lungs of mice infected with murine pneumonitis virus.*

Exp. no.	Group	Treatment*	Days after infection	Virus titer†
III	A	None	7	9.3
	E	Thyroxine	7	9.5
	A	None	9	13.1
	E	Thyroxine	9	12.8

* See text and table 2 for details of treatment with thyroxine.

† Virus titer is expressed as the mean survival time of groups of 15 mice infected in the cloud chamber with lung pools prepared as described in the text. These titrations were performed by Mr. Frank M. Gogolak. The detailed titration procedure will be described in another publication.

gether with control animals of the same age group, were exposed to an aerosol of murine pneumonitis virus of about the concentration employed in experiments I and II. As seen from figure 4 and table 2, administration of a single dose of 0.08 mc radioactive iodine had no effect upon the susceptibility of mice to murine pneumonitis.

The mice used in experiment V were a month old and weighed about 15 g. They were given a single subcutaneous injection of 0.1 mc radioactive iodine in 0.1 ml 0.9% sodium chloride, and as in experiment IV were infected in the cloud chamber three months after injection of the radioactive iodine. The mice receiving this larger dose of radioactive iodine lived significantly longer than untreated animals (fig. 5, table 2). The one survivor of the treated group was killed on the 34th day. Although its lungs were extensively consolidated this mouse was excluded from calculation of the mean survival time of its group. All the other mice of experiment V exhibited complete or nearly complete lung consolidation upon autopsy.

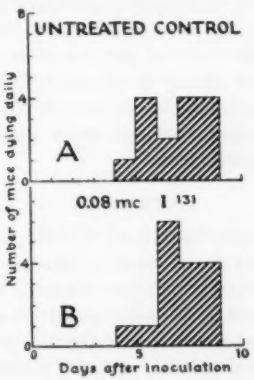

FIG. 4.—Effect of radioactive iodine I^{131} on the survival time of mice infected with murine pneumonitis (exp. IV).

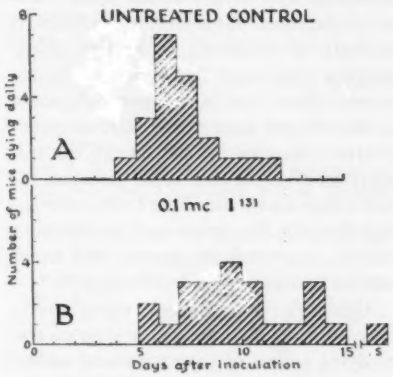

FIG. 5.—Effect of radioactive iodine I^{131} on the survival time of mice infected with murine pneumonitis (exp. V).

The results of experiments IV and V show that dosage with 0.1 mc radioactive iodine caused destruction of the thyroid tissue and a prolongation of the life of mice with murine pneumonitis, although a slightly smaller dose of 0.08 mc, while causing about the same amount of thyroid destruction, had no effect upon the survival time of the infected mice. Further work on the relation between amount of radioactive iodine given and effect on resistance is obviously needed before a clear picture can be obtained.

DISCUSSION

These results clearly indicate that the resistance of mice to air-borne murine pneumonitis may be decreased by thyroxine and increased by radioactive io-

dine. The effect of these substances on resistance to infection is most logically explained in terms of their effect on thyroid activity. Histological studies showed that the thyroid glands of the treated mice were affected in the expected manner, and it is highly unlikely that either thyroxine or radioactive iodine had any direct effect upon virus multiplication. However, the observed changes in resistance to murine pneumonitis virus must be attributed to unknown alterations in the metabolic state of the infected mice rather than to quantitative changes in the energy metabolism of the infected cells, because intact lungs and liver slices from mice injected with thyroxine and radioactive iodine consumed oxygen at the same rate as tissues from normal animals.

Although the present experiments give only an incomplete picture of the mode of action of thyroxine and radioactive iodine, certain conclusions may be drawn concerning their effect on resistance. Thyroxine does not increase the growth rate of murine pneumonitis virus and thus hasten the death of the treated mice. On the other hand, thyroxine cannot act solely by increasing the susceptibility of the infected animals to the lethal action of the virus or by subjecting both the infected and uninfected cells of the lungs to a heightened metabolic strain due to the increased metabolic rate, although both effects may play some part in decreasing the survival time of thryoxine-treated mice. It is true that some of the thyroxine-treated mice which died early had incompletely consolidated lungs, but the concept of premature death without alteration of the course of infection cannot explain all of the results. Mice receiving thyroxine late in the course of infection lived just as long as control mice. Also, it is unlikely that the increased survival time of mice treated with radioactive iodine is due merely to a lesser metabolic demand on the lungs because the lungs of both treated and control mice were completely or almost completely consolidated at death. Therefore, it appears that thyroxine and radioactive iodine do not simply change the metabolic rate of infected mice and thereby change the metabolic requirements for survival, but instead play a more fundamental role in resistance to infection.

The results of experimental procedures which in some way injure the host and cause a decrease in its resistance to infection should be interpreted with great caution. Therefore, the experiments with radioactive iodine are especially valuable in that they afford a further control for the thyroxine experiments. Although radioactive iodine caused greater tissue changes, and undoubtedly greater general damage to the mice, it prolonged their survival time after infection with murine pneumonitis virus. This indicates a specific role for radioactive iodine and at the same time suggests that the effect of thyroxine in shortening the survival time cannot be attributed solely to nonspecific injury to the mice.

In conclusion, the relation of thyroid activity to susceptibility to infection with the virus of murine pneumonitis has been clearly demonstrated, but an explanation of the precise role played by the thyroid hormone must await further investigation.

SUMMARY

The relationship of thyroid activity to resistance of mice to infection with the virus of murine pneumonitis was investigated by the administration of thyroxine which increased thyroid activity and of radioactive iodine I^{131} which completely or almost completely destroyed the thyroid.

Subcutaneous injection of 0.1 to 0.2 mg thyroxine on alternate days significantly lowered the survival time of mice infected with murine pneumonitis virus in a cloud chamber. The administration of thyroxine was begun 8 to 14 days before infection or on the day after infection with a more dilute inoculum. Thyroxine given late in the infection, beginning with the seventh day after exposure to the virus, did not alter the survival time of the mice.

In contrast to thyroxine, a single subcutaneous injection of 0.1 mc radioactive iodine per mouse increased the resistance of the mice when they were infected with murine pneumonitis virus three months later. A single injection of 0.08 mc radioactive iodine had no effect.

Intact lungs and liver slices from thyroxine and radioactive iodine-treated mice consumed oxygen at the same rate as tissues from untreated animals. Therefore, the observed changes in resistance to murine pneumonitis appear to be due to unknown alterations in the metabolic state of the infected animals rather than to simple quantitative changes in the level of energy metabolism of the infected cells. An understanding of the mechanisms involved requires further investigation.

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RESPIRATORY INFECTION IN THE RABBIT: AN ENZOOTIC CAUSED BY *PASTEURELLA LEPISEPTICA* AND ATTEMPTS TO CONTROL IT BY VACCINATION

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The existence of a respiratory disease in domestic rabbits is a problem of concern not only to the laboratory but also to the commercial breeder. The disease, known commonly as "snuffles," has been recognized since rabbits were first used as laboratory animals. The etiology of the disease has received extensive investigation by several workers, chief among them being Ferry,[1] who isolated an organism associated with the disease which he called *Bacillus bronchosepticus*, and Webster,[2–4] who concluded that the causative organism was primarily *Pasteurella lepisepticum*.

The present investigation was undertaken to determine the cause of a 50% mortality rate, with death almost invariably due to pneumonic infection or accompanied by it, in a colony consisting of about 1000 rabbits, many of which are inbred. The etiology of similar respiratory diseases in rabbits shipped from breeders and laboratories in other localities was also determined.

It has been shown by De Kruif[5] that two rabbits were rendered immune by the injection of killed *Past. lepiseptica*. Ferry and Hoskins[6] have also demonstrated protection in rabbits by vaccination with *Brucella bronchoseptica*, but not with *Past. lepiseptica* in the dosage employed. Because of the value of the animals and the severity of the enzootic, the efficacy of vaccination against the organism isolated in the Jackson Laboratory colony was determined.

EXPERIMENTAL

Isolation of the organism.—An organism was isolated in pure culture from 4 rabbits who had manifested typical respiratory symptoms prior to death. The lung was aseptically removed in each case, and a piece approximately 1 cm square was cut from the area showing evidence of pneumonia. This was impressed upon 5% rabbit's blood agar and the culture incubated for 24 hours at 37 C.

The gram-negative bacillus as isolated is described in table 1, in which it is compared to *Past. lepiseptica* and to *Br. bronchoseptica*.

From table 1 it can be seen that the organism isolated here is very similar to *Past. lepiseptica*. It is also similar to strains of this organism described previously by Ferry and Hoskins[6] and De Kruif.[5] Apparently it does not, however, follow the form of variation as observed by De Kruif. The mucoid, translucent, iridescent form, invariably virulent and invariably growing only diffusely in broth, is the only form in which the organism has been isolated here. De Kruif[5] described *D* and *G* forms, growing diffusely and in settled granular deposits

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TABLE 1.—*Differential comparison of strains of gram-negative bacilli known to cause pneumonia in the rabbit.*

	Br. bronchoseptica	*Past. multocida (lepiseptica)*	Jackson laboratory
Source	#780 American Type Culture Collection	#7288 American Type Culture Collection	Rabbit lung
Differential morphology:			
Cellular	Coccobacillus, marked pleomorphism	Bacillary, larger, very slight pleomorphism	Coccobacilli, sometimes encapsulated, occasional long threadlike form
Colonial	Opaque	Mucoid, translucent	Mucoid, translucent
Blood agar	Hemolytic	Nonhemolytic	Nonhemolytic
Plain agar	Not iridescent	Iridescent	Iridescent
Motility	Motile	Nonmotile	Nonmotile
Utilization of sugars:			
Dextrose	0	+	+
Maltose	0	0	0
Mannite	0	+	+
Lactose	0	0	0
Pathogenicity for mice	Death <24 hours, lungs congested, turbinates inflamed	Death 1–8 days, intestinal congestion, slight respiratory involvement	Death 1–7 days, intestinal congestion, slight respiratory involvement

and being virulent and avirulent, respectively.

This microorganism is the only one which has been isolated from the lungs, trachea, or turbinates of animals dead of respiratory disease in this colony, and it has invariably been in pure culture.

The disease was reproduced by the intravenous injection of 5 ml of the organism grown on blood agar, suspended in saline and made to a turbidity of T-20 at 450 γ on a Junior Coleman Spectrophotometer. Two rabbits, one 3 months old and the other 6 months old, were injected. Litter mates of these animals served as controls and received the exact preparation, except that the organisms had been killed by heating for 10 minutes in a boiling water bath. The younger animal died in less than 18 hours, the older in 4 days. Neither of the control animals ever showed any symptoms of respiratory disease. A pure culture of the injected microorganism was recovered at autopsy from the lung and trachea of both experimental animals.

The organism has been isolated from every case of the disease. It has been isolated by nasal swabbing from live rabbits which were apparently healthy, but these animals developed the disease within one to two weeks. This is contrary to the observations of De Kruif[7] and Webster,[3] who believe that a carrier state exists. This may be partially explainable on the basis of the exclusive occurrence of the virulent organism in this rabbit colony.

Etiology of rabbit pneumonia from other colonies.—A total of 12 rabbits manifesting mild or severe symptoms of the disease were sent in from 7 states. After observation for a length of time dependent on the severity of the symptoms, the rabbits were killed by an air embolus. The lungs, trachea, and turbinates were cultured as before. The organisms which were isolated were studied to determine whether or not they were either *Past. lepiseptica* or *Br. bronchoseptica*. Of the 8 animals which were chronically ill, *Past. lepiseptica* was isolated from 5 and *Br. bronchoseptica* from 3. The 4 acutely ill animals were all infected with *Past. lepiseptica*.

First vaccination experiment.—The 18-hour growth of the organism, freshly isolated from a rabbit dead of pneumonia, was washed from blood agar with sterile saline. The turbidity was adjusted to a reading of approximately T-20 as before. One-half of the suspension was heated in a boiling water bath for 10 minutes; anaerobic and aerobic cultures were made to assure sterility of the vaccine. Twelve rabbits were chosen for vaccination, and 9 rabbits of similar age served as controls. Animals were

7. De Kruif, P. H. 1922, J. Exper. Med. **36**: 309–316.

observed for a period of 2 weeks prior to injection to insure the absence of disease. All animals were segregated from the ordinary stocks and were kept in outdoor cages. The vaccine was administered intravenously in a total quantity of 10 to 15 ml over a period of 3 weeks. Seven to 10 days following the last injection, the immunity of the animal was challenged by the intravenous injection of living organisms. Two challenging doses were arbitrarily selected: 2.5 and 5.0 ml of a live suspension prepared similarly to and adjusted to the turbidity of the vaccine. The animals were examined daily for appearance of symptoms. If death occurred, the animal was autopsied immediately and the lungs, trachea and turbinates cultured as before.

Of the 12 vaccinated animals, 11 survived without ever showing symptoms of the disease; one animal died in less than twenty-four hours, and the organism was recovered at autopsy. Seven of the 9 unvaccinated animals died within 10 days; all showed respiratory lesions and the organism was recovered in pure culture. The 8th animal died of diarrhea and the organism was not recovered; the 9th was sacrificed on the 21st day and the organism was not recovered.

Second vaccination experiment.—All rabbits in the colony which were born between arbitrarily selected dates were used in this experiment. Animals over 2 months old which showed no symptoms of respiratory disease and which were not pregnant were divided into two groups. One group, comprising 184 animals, was vaccinated as follows: 1 ml for 2 days; rest 5 days; 2 ml for 2 days; rest 5 days; 3 ml for 2 days. The other group, comprising 522 animals, served as controls. Insofar as possible, both groups included representatives of all of the inbred races. Exposure was left to chance. Animals in both groups were treated identically, and no attempt was made to cause any interruption of the ordinary routine. When death, which was taken as the end point, occurred, the animals were autopsied. If there was evidence of respiratory infection, even though there was evidence of other infectious processes not necessarily associated with the disease, death was attributed to respiratory infection. Results of this experiment are recorded in table 2, from which it can be seen that in a total of 706 animals, death due to respiratory disease was lowered from 14.75% to 8.69% by the administration of the vaccine.

DISCUSSION

The occurrence of gram-negative bacilli as the etiological agents in acute respiratory diseases of domestic animals is well known. Despite attempts to con-

TABLE 2.—*Results of immunization of rabbits in field trial.*

Race	Vaccinated			Not vaccinated		
	Number of animals	Number dead having respiratory pathology	Percent dead	Number of animals	Number dead having respiratory pathology	Percent dead
X	43	5	11.6	44	5	11.3
III-c	37	5	13.5	44	5	11.3
III	16	1	6.25	15	3	20.0
Hyde	1	0	0	7	7	100
KD	5	0	0	10	1	10
DRD	11	0	0	31	1	3.2
Ach	4	0	0	57	12	21
OS	8	0	0	20	1	5
Rous	2	0	0	2	0	0
V	4	0	0	8	2	25
Sandy	5	0	0	8	3	36.5
Misc.	48	5	10.4	276	37	13.4
Total	184	16	8.69	522	77	14.75

$X^2 = 5.25$, $P = < 0.02$.

trol it by sanitation, mortality due to such diseases, particularly in the rabbit, is still high and is a serious problem to laboratory personnel as well as to the commercial breeder. The practice of sacrificing infected rabbits may cause a serious economic loss to the breeder and a serious experimental loss in the laboratory. The present investigation serves to re-emphasize the importance of this group of microorganisms in animal economy.

A virulent strain of *Past. lepiseptica* has been demonstrated to be the sole cause of pneumonia in a large colony of domestic rabbits. This does not exclude the possibility, however, that other organisms, such as *Br. bronchoseptica*, which has been isolated from rabbits in other colonies, also contribute to respiratory disease. The syndrome known familiarly as "snuffles" may or may not be an entity, but in either case it and other similar respiratory infections of the domestic animal are deserving of investigation to the end of devising a means of prevention.

The results of vaccination with a strain of *Past. lepiseptica* isolated within the colony show that there is definite but not invariable protection against death when a heat-killed organism is injected. On examination of table 2 it can be seen that rabbits of race X and race III-c are apparently not benefited by the vaccine, while those in race III and possibly race V are. The reason for this might well lie in the basic genetic difference between the races. It has been observed in unpublished experiments made in this laboratory that individual members of race III and race V almost always produce a large quantity of antibody against an injected antigen, while it is well known that the average rabbit may not.

In rabbit colonies in which valuable animals are housed, the use of the vaccine, although it does not afford 100% protection, would appear to be advantageous. The opinion is held at this laboratory that the vaccine was in large part instrumental in saving race III from extinction during a severe enzootic of respiratory infection. This is based on vaccination of other members of the race after the results expressed in table 2 had been tabulated.

In the ordinary laboratory colony, the vaccine could be advantageous in a situation in which respiratory disease was introduced into the colony by the importation of an infected rabbit. The organism isolated from that rabbit could be used as a source of antigen and a vaccine prepared and administered to the remaining members of the colony. This could ward off a possible enzootic or the probable establishment of the disease in the colony.

SUMMARY

1. A gram-negative bacillus, probably a strain of *Pasteurella lepiseptica*, has been established as the cause of a respiratory infection in a rabbit colony composed of 1000 rabbits, a large proportion of which are inbred.

2. Either *Past. lepiseptica* or *Brucella bronchoseptica* was isolated from infected rabbits from other states.

3. A vaccine has been prepared from the organism isolated within the large colony which is demonstrably protective when death is taken as the end point when exposure is known or when exposure is left to chance.

4. It is suggested that the variation in protection afforded members of the different races is due to the difference in genetic constitution of the races of rabbits.

5. It is suggested that the use of a vaccine would be advantageous in breeding colonies of rabbits, as well as to the laboratory colony which is free of infection, to prevent the establishment of the disease from a newcomer to the colony.

STUDIES OF THE EFFECT OF ANTIBIOTICS ON INFECTIONS WITH THE MOUSE PINWORM *ASPICULURIS TETRAPTERA*

II. THE ACTIONS OF NEOMYCIN, DIHYDROSTREPTOMYCIN AND CHLORAMPHENICOL

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In a previous report (Wells, 1951) the findings of the effect of terramycin hydrochloride on experimental infections with the mouse pinworm *Aspiculuris tetraptera* were presented. The results showed that the worm burden in mice was significantly reduced and the remaining worms were stunted in size. Successful therapeutic trials in man with terramycin (Wells et al, 1951; Loughlin et al, 1951) have demonstrated that *A. tetraptera* infections in mice are of value in screening new drugs for enterobiasis. In this report our findings with neomycin, dihydrostreptomycin and chloramphenicol are presented.

METHODS AND MATERIALS

The same techniques were employed here as in the previous study (Wells, 1951). Helminth-free mice were fed approximately 300 infective eggs of *A. tetraptera* by stomach tube. Treated mice received single, oral, daily doses of neomycin at the rate of 50 mg/kg. Other mice received 500 mg/kg of dihydrostreptomycin by the same route. Oral dosage of 500 mg/kg was employed with chloramphenicol. All the treated mice and controls were sacrificed 14 days after infection. The entire large intestines were examined microscopically and the worms were counted. The length of worms recovered was measured in the experiments with dihydrostreptomycin and chloramphenicol.

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We wish to thank the Upjohn Co., Kalamazoo, Mich. for providing the neomycin used in this study.

The author wishes to extend thanks to Dr. H. W. Brown for advice in conducting these experiments and preparation of the manuscript.

EXPERIMENTAL

Neomycin

Experiment 1.—Treated mice received 50 mg/kg of neomycin in single daily oral doses for 8 days beginning 6 days after infection. The day after receiving the last dose the mice were autopsied and the worms were counted (i.e., 14 days after infection). The average worm yield in the control mice was 48 and in the treated mice 108, a highly significant difference. (See table 1.)

Experiment 2.—Mice received 17 daily doses of neomycin, 3 of which were administered prior to infection. On autopsy 14 days after infection, the control mice harbored on the average 47 worms and the treated animals 111, a highly significant difference.

Experiment 3.—Treated mice received 7 daily doses of neomycin beginning 7 days after infection. On autopsy 14 days after infection, the control mice, which were the same as in experiment 2, harbored an average of 47 worms and the treated animals 68, a difference which proved to be significant.

Experiment 4.—This experiment was set up to confirm further the surprising results in the previous three experiments and is a duplicate of experiment 2. After autopsy the average worm yield in control mice was 55 and in treated mice 116, a highly significant difference.

TABLE 1.—*The effect of neomycin on* A. tetraptera *worm burden in mice.*

Exp. no.	Number of mice	Treated animals			Control animals		
		Day of infection treatment was initiated	Total days of treatment, 50 mg/kg daily	Average worm yield	Number of mice	Average worm yield	t*
1	15	6	8	108	15	48	6.7†
2	14	−3	17	111	15	47	7.8†
3	15	7	7	68	15	47	2.7†
4	15	−3	17	116	15	55	7.7†

All animals received 300 eggs.
* Difference in means/standard error of the difference in means.
† Indicates significant difference ($P < 0.01$) between treated and control animals.

Dihydrostreptomycin

Experiment 5.—Treated mice received 16 daily doses of dihydrostreptomycin at the rate of 500 mg/kg beginning 2 days prior to infection. On autopsy 14 days after infection, the control mice harbored an average of 99 worms and treated animals 171, a highly significant difference. (See table 2.)

Experiment 6.—Treated mice received 7 daily doses of dihydrostreptomycin beginning 7 days after infection. On autopsy 14 days after infection, the average worm yield in control mice was 99 and in treated mice 121, a difference which did not prove to be significant on statistical analysis.

Experiment 7.—This experiment is a duplicate of experiment 5. Treated animals received 16 daily doses of dihydrostreptomycin beginning 2 days prior to infection. On autopsy 14 days after infection control mice harbored an average of 52 worms and treated mice 111, a highly significant difference.

Experiment 8.—In bacteriological studies (unpublished) of the fecal flora, we found that with dihydrostreptomycin therapy there was a decline in total aerobic counts and almost a complete disappearance of coliform organisms. This confirms a previous report of Smith and Robinson (1945). By the 14th day of therapy however, coliform counts began to increase. We do not know whether the antibiotics affect the worms directly or act through their alteration of intestinal flora. We wondered whether there was any relation between the increased worm burden and the alteration in total aerobic and coliform counts. In this experiment, therefore, we administered 14 daily doses of dihydrostreptomycin prior to infection and continued treatment for 14 days after infection, a total of 28 days of therapy. This experiment was set up simultaneously with experiment 7 so that treated and control animals in the latter served as controls. On autopsy 14 days after infection, the average worm yield in treated animals was 100 as compared with 52 in control animals, a highly significant difference. The difference in worm burden in

TABLE 2.—*The effect of dihydrostreptomycin on* A. tetraptera *worm burden in mice.*

Exp. no.	Number of mice	Treated animals			Control animals		
		Day of infection treatment was initiated	Total days of treatment, 500 mg/kg daily	Average worm yield	Number of mice	Average worm yield	t*
5	15	−2	16	171	15	99	4.9†
6	15	7	7	121	15	99	1.5
7	15	−2	16	111	14	52	5.5†
8	14	−14	28	100	14	52	4.3†

All animals received 300 eggs.
See footnotes under table 1.

treated animals in experiments 7 and 8 was not significant. This indicated that the reappearance of coliform organisms had no apparent effect on the worm burden.

In experiments 5 through 8, lengths of worms recovered at autopsy were measured. In no instance was there a significant difference in size between worms from treated and untreated, control animals.

Chloramphenicol

Experiment 9.—Treated mice received 16 daily doses of chloramphenicol at the rate of 500 mg/kg beginning 2 days prior to infection. On autopsy 14 days after infection, control mice harbored an average of 69 worms and treated mice 131, a highly significant difference. (See table 3.)

TABLE 3.—*The effect of chloramphenicol on* A. tetraptera *worm burden in mice.*

Exp. no.	Number of mice	Treated animals			Control animals		P*
		Day of infection treatment was initiated	Total days of treatment, 500 mg/kg daily	Average worm yield	Number of mice	Average worm yield	
9	15	−2	16	131	15	69	3.9†
10	15	7	7	99	15	69	2.1
11	15	−2	16	153	15	91	3.5†

All animals received 300 eggs.
See footnotes under table 1.

Experiment 10.—Treated mice received 7 daily doses of chloramphenicol beginning 7 days after infection. On autopsy 14 days after infection the average worm yield in control animals was 69 and in treated animals 99, a difference which proved to be of borderline significance on statistical analysis.

Experiment 11.—This experiment is a duplicate of experiment 9. Treated animals receiving 16 daily doses of chloramphenicol harbored an average of 153 worms, while controls harbored an average of 91, a significant difference.

In experiments 9 through 11 lengths of worms were measured. There was no significant difference in size between worms from treated and untreated, control animals.

DISCUSSION

From the above data it is evident that a proportion of larvae are lost during the course of their development to the adult stage. In neomycin-, dihydrostreptomycin- and in chloramphenicol-treated mice, a greater proportion of worms continued development in the colon than in untreated mice.

A. tetraptera is a small nematode of the colon, found primarily just below the ileocecal region. We have never found the worm embedded in the mucosa in histological sections. The alimentary tract of the worm contains colonic material including bacteria and trichomonads, although the possibility that the worms ingest small fragments of superficial mucosa cannot be eliminated. The worm appears to ingest intestinal contents indiscriminately as evidenced by its ingestion of carmine particles fed to the mouse. Coliform bacilli are considered to be the major synthesizers of members of the vitamin B complex in the intestine (Gant et al, 1943). In the present study with two antibiotics, dihydrostreptomycin and neomycin, that suppress or eliminate coliform organisms, there was a surprising phenomenon of a coexisting favorable environment for the mouse pinworm. Moreover, as shown in experi-

ment 8, a reappearance of resistant coliform organisms during dihydrostreptomycin therapy had no relationship to the increase in worm burden. There are indications that resistant flora differ from parent strains. Miller (1945) demonstrated that resistant coliform strains do not synthesize comparable amounts of folic acid as compared with the strains from which they were derived. Therefore, the hypothesis of the action of dihydrostreptomycin through its effect on fecal flora has not been eliminated.

In bacteriological studies (unpublished) of the fecal flora of mice receiving chloramphenicol therapy, we found considerable variation in the response of coliform organisms. In general, however, coliform counts were increased. Though alterations in the fecal flora with chloramphenicol appear to be different from those of either neomycin or dihydrostreptomycin, the effect on the worm is similar. We have no proof that a favorable environment is created for *Aspiculuris* through a change in the flora.

Terzian (1950) demonstrated that when sulfonamides are added to the maintenance diet, there is increased susceptibility to infection of *Aëdes aegypti* with *Plasmodium gallinaceum*. He found that with a specific optimal drug concentration a significantly greater number of oöcysts developed in mosquitoes receiving sugar solution containing sulfonamides than in controls. The author suggested that depriving the host of its available para-aminobenzoic acid disturbs the innate immunity of the mosquito and heightens the level of infection in the normal host. We cannot offer the hypothesis that the host is suffering some nutritional lack to explain the increased worm burden during treatment with these antibiotics. The mice were maintained on a balanced diet, continued to gain weight and showed no evidence of toxic reactions. Moreover, in some unpublished studies with sulfadiazine we found that while there was no alteration in the worm burden, a significantly smaller worm was obtained in treated animals.

All the antibiotics we have studied have some action on *A. tetraptera* infections in mice. They either increase the worm burden or suppress the infection. We cannot offer any explanation of these effects. The mechanism of action may perhaps be revealed when in vitro cultivation of parasitic nematodes becomes possible.

SUMMARY

1. The effects of neomycin, dihydrostreptomycin and chloramphenicol on infections with the mouse pinworm *Aspiculuris tetraptera* have been presented.

2. Administration of these drugs results in an increase in the worm burden as compared with the controls.

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STUDIES ON THE BIOLOGY OF *CRYPTOCOCCUS*

IV. ISOLATION OF A HIGHLY POLYMERIZED POLYSACCHARIDE FROM ENCAPSULATED STRAINS

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The polysaccharide components of encapsulated yeasts have been isolated previously by heating whole cultures[1,2] or saline suspensions of cells[3] and separating the polysaccharide from this crude extract by alcoholic precipitation. The physical characteristics of polysaccharide isolated in this laboratory by acid extraction or by extraction with 2,4-dinitrophenylhydrazine in acid* differed markedly from the viscous, mucoid appearance of whole cultures of *Cryptococcus neoformans*. Since the mucoid character of whole cultures of encapsulated cells suggested the presence of highly polymerized structures, extraction with hot alkali, which may produce swelling and solubilization of alkali-resistant polymers, was attempted. The present report is presented as a basis for further improvement in the isolation and characterization of the capsular polysaccharide of this species.

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* This method of extraction was suggested by Dr. Maurice Pechet, of these laboratories.

METHODS

Two strains of *C. neoformans* recently isolated from human cases of cryptococcosis were used. Both strains were in the M-phase[4] but differed in degree of encapsulation. One strain was characterized by large, broad capsules and a mucoid, confluent growth, while the other presented smaller but distinct capsules and drier, less mucoid colonies on Sabouraud's agar. Although a few poorly encapsulated cells usually could be found in India ink preparations of the former, heavily encapsulated cells were seen rarely in similar preparations of the latter (fig. 1). Both strains were pathogenic for mice, and, as has been reported by others,[5] differences in the degree of encapsulation were not as marked in material from infected mice.

Ammonium sulfate-glucose broth (with thiamine hydrochloride, 0.2 γ/ml), as used by Mager,[1] Hehre et al[2] and Mager and Aschner,[4] was dispensed in 500 ml volumes in one-liter Erlenmeyer flasks, inoculated with heavy suspensions of these strains and incubated at 25 C for 10 days with constant aeration. The cultures were checked for purity, centrifuged, the cells washed 3 to 4 times with saline, packed at 5000 g and drained of supernatant.

The cells were heated with an equal volume of 8*N* sodium hydroxide for 20 minutes in a boiling water bath with constant stirring, cooled, neutralized with 6*N* hydrochloric acid and centrifuged at 2000 g. The supernatant was collected, brought to pH 4.5 with 6*N* hydrochloric acid, recentrifuged and the precipitate discarded. Two

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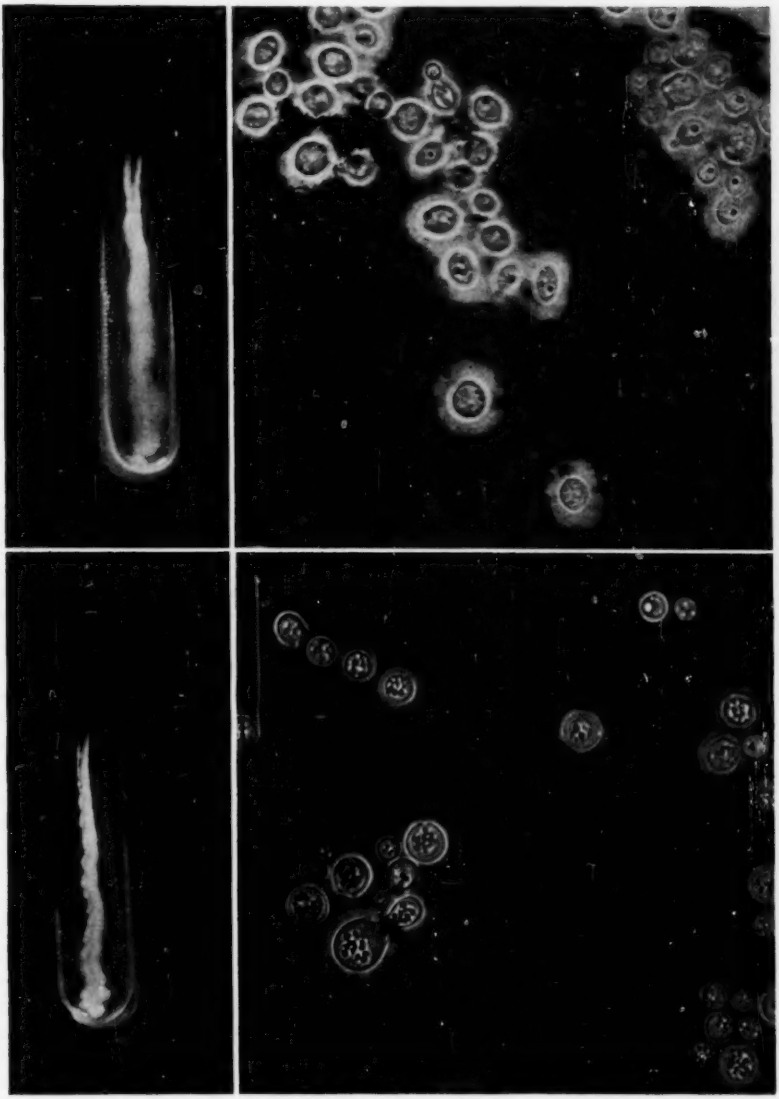

FIG. 1.—Morphological characteristics of strains used. Note confluent growth of heavily encapsulated strain (top) and drier, nonconfluent growth of strain with smaller capsules. Sabouraud's agar, 7-day cultures. India ink capsule preparations. ×1100.

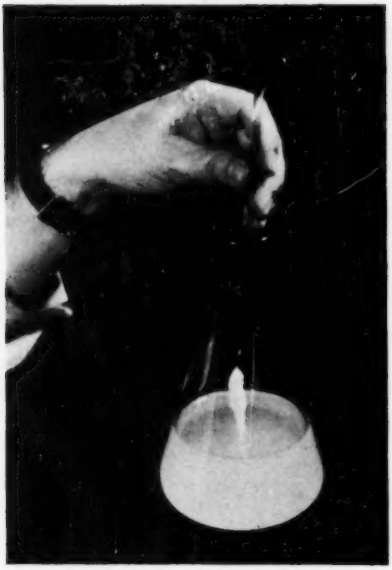

FIG. 2.—Collection of white, stringy flocculate resulting from alcoholic precipitation.

volumes of 10% trichloracetic acid were added to this supernatant, the mixture was immediately centrifuged in the cold at 5000 g and the precipitate discarded. The white, opalescent supernatant thus obtained was precipitated with 1.5 volumes of cold 95% ethanol. The resulting white, stringy flocculate was collected by winding around a glass rod (fig. 2), dissolved in water with dilute alkali added to pH 7.0 and dialyzed for 6 hours against running tap water. Yields varied from 133.5 to 210 mg per liter of medium in different experiments.

RESULTS

The resulting nondialyzable, opalescent, very viscous solution gave a strong reaction with Molisch's and Bial's reagents. No reaction was obtained with Ehrlich's reagent. Treatment with Lugol's solution did not elicit a positive reaction for glycogen or starch.† The polysaccharide lost its solubility upon drying in a desiccator or upon lyophilization. Hence only known aliquots were taken to dryness for the determination of solids, nitrogen (micro-Kjeldahl), phosphorus[6] and the study of hydrolytic products.

The nitrogen content varied between 0.20 to 0.24%. Preparations having a higher initial nitrogen content were further purified by treatment with 1 volume of 10% trichloracetic acid, centrifuging at 4000 g and precipitating the supernatant with 1.5 volumes of cold 95% ethanol. The precipitate was redissolved in water, shaken with an equal volume of chloroform, the supernatant aqueous layer reprecipitated with 1.5 volumes of cold 95% ethanol and the ensuing white, stringy precipitate collected and dissolved in water. None of the preparations thus obtained contained phosphorus.

Aliquots of the dried polysaccharide were hydrolyzed with 6*N* hydrochloric acid under nitrogen in sealed tubes at 110 C for 8 hours. The hydrolysates thus obtained contained a large residue of dark brown, insoluble material. The acid was removed by repeatedly taking the hydrolysates to dryness in vacuo. The solid residue was then extracted with water and subjected to paper partition chromatography, using phenol-water and butanol-acetic acid for one- and two-dimensional runs. Amino acids could not be detected in any of these preparations. Glucosamine likewise could not be detected. Thus the nitrogen content of these preparations could not be ascribed to contaminating amino acids or the presence of glucosamine in the polysaccharide.

Complete acid hydrolysis as described above did not yield reducing substances as determined by a micromodification of

† Both strains produced the amylose (fraction A) described by Mager[1] and Hehre et al,[2] which was isolated according to the procedure described by Hehre et al.

6. Fiske, C. H. and Subbarow, Y. 1925, Colorimetric determination of phosphorus. J. Biol. Chem. **66**: 375–400.

Hanes' method[7] or by the method of Folin and Malmros.[8] Mild acid hydrolysis (1*N* hydrochloric acid for 1 hour at 100 C) gave a colorless insoluble residue, while the supernatant was found to contain 0.5% reducing substance (calculated as glucose). The insoluble residue formed stained intensely blue during the course of the Folin-Malmros reaction. Further hydrolysis, however, failed to show the liberation of additional reducing substance, probably due to breakdown occasioned by the process.

Relative viscosity was determined at 25.5 C with Ostwald-Fenske viscosimeters on 0.1, 0.2, 0.4 and 0.6% solutions of the polysaccharide and the intrinsic viscosity [η] calculated by extrapolation to infinite dilution. In 4 preparations thus studied the intrinsic viscosity was found to vary between 16.0 and 27.7 (table 1).

Treatment of the polysaccharide solutions with commercial preparations of hyaluronidase (Alidase, Hydase) did not produce any measurable change in viscosity. Similarly, incubation of viable cells of these strains at 25 and 37 C in the presence of 1.0 to 300.0 T.R.U. in acetate buffers at pH 4.5 and pH 6.0 or in phosphate buffers at pH 6.0 and pH 7.0 for intervals varying from 5 minutes to 24 hours failed to show any evidence of decapsulation.

Preliminary attempts to elicit precipitins, agglutinins or sensitization in mice, guinea pigs and rabbits with these polysaccharide preparations have been negative. Moreover, with antiserums produced with vaccines of intact heat-killed or formalinized cells of homolo-

TABLE 1.—*Characteristics of capsular polysaccharides isolated from cryptococcus.*

Reference	Strain	Incubation	Starting material	Method of extraction	Viscosity	Nitrogen	Phosphorus	Amino acids	Reducing substance*
Mager[1]	*T. rolanda*	4–5 days	Whole culture	60–80 C, 30 min.	Thread-like	0.00	Not stated	0.00	58.0–67.0%
Klugman*†	*C. neoformans*	14 days	Cells	75 C, 35 min., 0.5*N* HCl	Colloidal	–0.10%	±1.50%	Not stated	0.00
Heinz[2]	*C. neoformans*	5 days	Whole culture	70–80 C, 30 min., pH 7.0, NaOH	Not stated	0.34%	Not stated	Not stated	65.00%
Present study	*C. neoformans*‡	10 days	Cells	25 C, 24–48 hr., 2,4-dinitrophenylhydrazine	Nonviscous	0.00	0.00	0.00	Trace
				70–80 C, 30 min., 0.5*N* HCl	Nonviscous	0.00	0.00	0.00	Trace
				100 C, 20 min., 8*N* NaOH	[η] = 16.0	0.20			
					18.2	0.32	0.00	0.00§	0.50%
					21.0	0.24			
					27.7	0.24			

* Calculated as glucose.
† Mentions that polysaccharide can be separated from cells disrupted by supersonic vibration but gives no description of product.
‡ The polysaccharides isolated from heavily encapsulated and moderately encapsulated strains appear to be identical.
§ Also negative by paper chromatography.

7. Hanes, C. S. 1929, Application of method of Hagedorn and Jensen to determination of larger quantities of reducing sugars. Biochem. J. **23**: 99–106.
8. Folin, O. and Malmros, H. 1929, Improved form of Folin's micro method for blood sugar determinations. J. Biol. Chem. **83**: 115–120.

gous or heterologous *Cryptococcus* strains, the alkali-extracted preparations gave either negative or very weak precipitin reactions that were demonstrable only with a 1:2 dilution of serum after 3 to 5 days' storage in the refrigerator. A product prepared by Hehre et al[2] and employed by Neill et al[5,9,10] for testing of antiserums represents the only purified cryptococcus polysaccharide whose serological reactivity has been reported in the literature.* Since it seemed possible that failure to obtain convincingly positive precipitation reactions with this polysaccharide might be due to the inadequate potency of the available antiserums, a sample was sent to Dr. J. M. Neill of Cornell University Medical College for tests against highly potent antiserums that were known to react with the cryptococcus polysaccharides prepared by Hehre et al.[2] This polysaccharide gave prompt precipitation with these more potent antiserums within 5 minutes at room temperature, in serum dilutions of 1:10 to 1:160. Further serological studies are being continued.

DISCUSSION

The polysaccharide described here differs from the fraction B described by Mager[1] and more recently by Hehre et al[2] in that it is highly polymerized and upon mild acid hydrolysis yields only a small amount of reducing substance, which appears to be destroyed with complete hydrolysis. It is interesting and possibly significant that the preparations which yielded a high percentage of reducing substance (as glucose) were obtained by the extraction of whole cultures 4 to 5 days of age, while the present preparations and those reported by Kligman[3] as yielding no reducing substance were obtained by the extraction of washed cells 10 and 14 days of age (table 1).

Alkaline extraction has been employed for the isolation of polysaccharide antigens from several species of bacteria.[11–15] As pointed out in a recent review of the subject,[15] alkali (or acid) frequently depolymerizes the polysaccharide, resulting in reduced viscosity. Since highly polymerized preparations were obtained by extraction with alkali, it appears that the polysaccharides from these strains are resistant to depolymerization by alkali whereas they are depolymerized easily by acid. This is supported by a comparison of the polysaccharides extracted with 0.5 *N* hydrochloride acid or 2,4-dinitrophenylhydrazine in acid in this laboratory, which were nonviscous and readily soluble in

9. Neill, J. M. and Kapros, C. E. 1950, Serological tests on soluble antigens from mice infected with *Cryptococcus neoformans* and *Sporotrichum schenckii*. Proc. Soc. Exper. Biol. & Med. **73**: 557–559.

10. Neill, J. M., Castillo, C. G., Smith, R. H. and Kapros, C. E. 1949, Capsular reactions and soluble antigens of *Torula histolytica* and of *Sporotrichum schenckii*. J. Exper. Med. **89**: 93–106.

* In addition to the reported product isolated from whole cultures, Hehre and Abrahams have prepared polysaccharides from the washed cells of a highly encapsulated *Cryptococcus* strain, which also have been proved to be serologically reactive (personal communication).

11. Krumwiede, C., Jr. and Noble, W. C. 1918, A rapid method for the production of precipitin antigen from bacteria: an attempt to apply it to the determination of type of *Pneumococcus* in sputum. J. Immunol. **3**: 1–10.

12. Kramár, E. 1921, Untersuchungen über die chemische beschaffenheit der kapselsubstanz einiger kapselbakterien. Centralbl. f. Bakt. 1. Abt. orig. **87**: 401–406.

13. Zinsser, H. and Parker, J. T. 1923, Further studies on bacterial hypersusceptibility. J. Exper. Med. **37**: 275–302.

14. Lancefield, R. C. 1925, The immunological relationships of *Streptococcus viridans* and certain of its chemical fractions. I. Serological reactions obtained with antibacterial sera. J. Exper. Med. **42**: 377–395.

15. Burger, M. 1950, Bacterial Polysaccharides, Springfield, Ill., C. C Thomas.

water even after drying, and the highly viscous alkali-extracted polysaccharide which loses its water solubility upon drying.

Viscosometric studies of polysaccharide treated with hyaluronidase under optimal conditions as well as the treatment of viable cells with hyaluronidase did not confirm the observation that this enzyme destroys the capsule of *C. neoformans*.[16] In view of these results it is unlikely that hyaluronic acid is a link in the polysaccharide chain itself although its presence as a branch of the main chain cannot be excluded.

16. Drouhet, E. and Segretain, G. 1949, Action de l'hyaluronidase sur la capsule de *Torula histolytica*. Compt. rend. Acad. d. sci. **228**: 424–425.

SUMMARY

An alkali-extraction method for the isolation of a highly polymerized polysaccharide from 10-day cultures of *Cryptococcus neoformans* is described. This product is Bial's positive, contains only a small amount of reducing substance, 0.20 to 0.24% nitrogen and no phosphorus. Nitrogen is present neither as glucosamine nor as amino acids. Intrinsic viscosity $[\eta]$ calculated by extrapolation to infinite dilution was found to be 16.0 to 27.7 for different preparations. These preparations are serologically active with appropriate antiserums. The recent observation that hyaluronidase destroys the capsule of *C. neoformans* could not be confirmed.

AGGLUTININS FOR SHEEP CELLS IN HUMAN SERUMS AND THEIR RELATIONSHIP TO THE A_2 ISOAGGLUTINOGEN-LIKE SUBSTANCE IN INFECTIOUS ORGANISMS

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When infectious organisms are incubated at 37 C in human serums of group B, the titer of the anti-A_2 isoagglutinin (α_2) is reduced to negative. The titers of the anti-A_1 (α_1) and anti-B isoagglutinin (β) in human serums of groups B and A, respectively, remain significantly unaltered. The organisms incubated in the serums of all blood groups included 10 species of bacteria, mature larvae of *Trichinella spiralis*, and adults of *Schistosoma mansoni*. Apparently, the bacteria and animal parasites contain and secrete a substance which has some immunological relationship to the A_2 agglutinogen of human erythrocytes, since it absorbs the homologous isoagglutinin.[1]

Since the infective organisms secrete the A_2 isoagglutinogen-like substance in vitro, it was of interest to determine whether such a substance would be secreted during infection of the host. This would imply testing of human serums during active or past infections for anti-A_2 agglutinin or other agglutinins which may be associated with it.

MATERIALS AND METHODS

The human red blood cells utilized in this study were obtained fresh and were washed three times in physiological saline previous to their use.* The sheep cells were preserved in Alsever's solution,† but were usually discarded two weeks after preservation. All blood serums used were heated at 56 C for 30 minutes.

For the agglutination tests 0.1 cc volumes of serum diluted 1:2, 1:4, etc. were mixed with 0.05 cc of the washed erythrocytes. The mixtures in Wassermann tubes were placed in a water bath at 37 C for two hours, refrigerated at 6 C overnight, and then examined microscopically for agglutinated erythrocytes. For convenience, the mixtures were examined while in the Wassermann tube, which was placed horizontally under the binocular dissecting microscope, under a magnification of ×20.

Serums to be absorbed with red cells were treated with 20% volume of the washed and packed erythrocytes. The suspensions were incubated at 37 C for one hour, centrifuged at 4000 r.p.m. for 10 minutes, and the supernatant treated again with fresh cells. Treatment of serum with fresh cells was performed three times after one hour incubation at each instance.

The adult worm materials to be added to the serum were prepared from recently obtained worms. These were dried in vacuo and well pulverized in an electric grinder. Enough of the material was added to serums to make a 1% suspension. The suspensions were incubated for one hour at 37 C and left overnight in the refrigerator at 6 C. The following morning these were centrifuged at 4000 r.p.m. for 15 minutes and the supernatant removed to be used as the absorbed serum.

Test for sheep cell agglutinins in human serums.—Blood samples were obtained from apparently healthy individuals who came to the blood bank as blood donors. Clinical histories revealed that most of these individuals had had some infectious disease at one time or another. Also, although no examination of the feces was done, there is a possibility that a number of them would have been infected with animal parasites, such as *Ascaris*, hookworm, and *S. mansoni*.

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1. Oliver-González, J. and González, L. M. 1949, J. Infect. Dis. **85**: 66.

* The blood samples were obtained at the time at which the donors at the blood bank of the School of Tropical Medicine were bled. The cooperation of Dr. Manuel Paniagua, head of the blood bank, and the laboratory technicians is acknowledged.

† Alsever's solution contains: dextrose, 2.05 g.; sodium citrate, 0.8 g; sodium chloride, 0.42 g; citric acid, 0.055 g.

Blood samples were also taken from individuals harboring microfilariae of *Wuchereria bancrofti*, as determined by examination of nocturnal blood. These individuals suffered no clinical symptoms as a result of the filarial infection. The infected individuals were bled during the morning from 9:00 to 10:00 a.m. The serums were removed after centrifugation and heated at 56 C for 30 minutes.

All of the serums were tested for sheep cell agglutinins at 37 and 6 C. In the case of the blood from patients infected with *W. bancrofti*, the serums were also tested for agglutinins to the individual's own cells (autoagglutinins).

Absorption of serums with erythrocytes, with living bacteria and animal parasites, and with dry well pulverized worm materials.—Serums found to be positive for sheep cell agglutinins at 37 C were treated with each of the following substances: (1) erythrocytes of human blood groups A_1, A_2, B, and O; (2) 48-hour cultures in tryptose agar of *Brucella abortus*, *Staphylococcus aureus*, and *Proteus OX19*; (3) known numbers of live *T. spiralis* larvae and adults of *S. mansoni*; (4) dry and well pulverized adult worm material from the human parasites *Ascaris lumbricoides* and *Necator americanus* and the dog filarial worm, *Dirofilaria immitis*. The serums were tested for sheep cell agglutinins before and after treatment with each of the above materials.

Human serums of group B previously tested for high titered anti-A_2 isoagglutinin were treated with the fresh erythrocytes from sheep cells. The treated and untreated serums were then tested for anti-A_2 agglutinin.

RESULTS

Agglutinins for sheep cells in human serums from apparently healthy individuals and from individuals harboring microfilariae of W. bancrofti.—The blood serums from 120 individuals were tested for sheep cell agglutinins, and of these, 98, or 81.7%, were positive. The titers varied from 1:4 to 1:128. All of the serums agglutinated the sheep cells at 6 C. The agglutinin titers at 6 C were considerably higher than those at 37 C for two hours. Titers varied from 1:64 to 1:1024 (table 1).

The serum of only one (3.9%) out of 26 individuals harboring microfilariae agglutinated sheep erythrocytes at 37 C. The titers of the agglutinins at 6 C were low, none exceeding 1:16 (table 1).

TABLE 1.—*Sheep cell agglutinins at 37 C and 6 C in serums from apparently healthy individuals and in individuals with circulating microfilariae of* W. bancrofti.

Number of serums tested	Agglutinins at 37 C		Agglutinins at 6 C	
	Number positive	Range of titers	Number positive	Range of titers
Serums from apparently healthy persons				
120	98 (81.7%)	1:4 to 1:128	120 (100%)	1:64 to 1:1024
Serums from persons with circulating microfilariae				
26	1 (3.9%)	1:4	20 (76.9%)	1:4 to 1:16

No agglutinins for the individual's own cells could be detected in the serums from the individuals with circulating microfilariae.

Absorption of sheep cell agglutinins by human subgroup A_2 erythrocytes and by dead and living materials from infectious organisms.—Five serums positive for sheep cell agglutinins were treated each with A_1, A_2, B, and O cells from three individuals of each blood group.

The titer of the sheep cell agglutinins in human serums at 37 C was reduced only after treatment with A_2 erythrocytes. Treatment with A_1, B, and O cells did not alter the titer of the agglutinins at all.

All of the serum samples into which the living bacteria or worms were introduced developed negative sheep cell agglutinin titers. Similarly, the sheep cell agglutinin titers were reduced to zero after treatment of the serum with dry and pulverized adult worm materials (table 2). Liver glycogen used as a control did not alter the agglutinin titers.

The agglutinins at 6 C were not absorbed by any of the substances introduced into the serums.

Absorption of anti-A_2 agglutinin by sheep erythrocytes.—Five serums of human group B were treated with sheep erythrocytes obtained from three different animals. The same serums were treated with erythrocytes obtained from

TABLE 2.—*Absorption of agglutinins for sheep cells in human serums by various living infectious organisms and by well pulverized adult worm material.*

Organism introduced into serum	Human serum blood groups	Sheep cell agglutinin titers at 37 C	
		Before treatment	After treatment
10,000 live larvae *T. spiralis*	A	1:64	0
10,000 live larvae *T. spiralis*	B	1:64	0
20 pairs adult *S. mansoni*	A	1:128	0
10 pairs adult *S. mansoni*	A	1:64	0
Staph. aureus	O	1:32	0
Br. abortus	O	1:128	0
Proteus OX19	O	1:128	0
Powdered adult *N. americanus*	B	1:128	0
Powdered adult *A. lumbricoides*	O	1:32	0
Powdered adult *Dirofilaria immitis*	O	1:32	0
Liver glycogen (control)	O	1:128	1:128

three different individuals of blood groups A_1, B and O.

The titer of the anti-A_2 isoagglutinin in human serum of group B was reduced to negative in all instances after treatment of the serum with sheep erythrocytes. The titer remained unaltered after treatment of the serums with A_1, B, and O erythrocytes.

DISCUSSION

The results obtained in this study suggest that the agglutinins for sheep red cells found in human serums (98 out of 124, or 81.7%) may be related to the presence of infectious agents at one time or another. As previously found, a large number of organisms when incubated in human serums secrete a polysaccharide-like substance which absorbs the anti-A_2 isoagglutinin in the serums.[1] The secretion or excretion of an isoagglutinogen-like substance, as observed in vitro, seems to take place also in vivo. This substance immunizes the host and leads to the production of antibodies. It is of great interest that this antibody can not be detected in the human serums as an anti-A_2 agglutinin as was to be expected, since there is an in vitro specific absorption of the anti-A_2 agglutinin by the substance in the infectious agent. Apparently, the substance in the organism has a complex structure, with part of it chemically related to the A_2 agglutinogen in human cells.

The fact that the secretions of the various live organisms and the worm material absorb the agglutinins for sheep cells at 37 C in the human serums suggest that the development of such agglutinins may be always a sequela of infection.

Agglutinins, as well as hemolysins for sheep cells, as found in human serums, are usually regarded as nonspecific antibodies, which react possibly due to cross reaction between antigens in the human and sheep erythrocytes. The findings of this study suggest that these antibodies are associated with infection, and that the agglutination of sheep cells is more likely due to the result of immunization with the substance in the parasite than to cross reactivity between the antigens in the two types of red cells.

The relationship between sheep red cell agglutinogens, the A_2 human blood substance, and antigens from bacteria and parasites is not understood at present. The three antigens have a common factor which absorbs the anti-A_2 and sheep cell agglutinins in human serums. The active substances in organisms associated with red cell agglutination or inhibition of agglutination seem to be polysaccharides alone, while the substance in sheep and human erythrocytes seems to be of a more complex nature.

Agglutination of human erythrocytes in vivo may be observed as a result of infection. As previously pointed out,[1] human erythrocytes with the A_2 substance may be agglutinated in vivo as a result of immunization with the A_2 isoagglutinogen-like substance present in infectious agents.

One out of 26 (3.9%) serums from the individuals with circulating microfilariae had agglutinins for sheep cells. The small percentage of serums positive for agglutinins among this group as compared to 98 out of 120 (81.7%) positive for agglutinins in the group of blood donors may be of significance. The destruction of numerous microfilariae or the secretions of the adult and embryos may constantly absorb the antibody formed. It is therefore likely that the serum may have an excess of parasite antigen rather than the antibody which develops as a result of immunization with it. This indicates, therefore, that tests should be performed to disclose antigen and not antibody.

SUMMARY AND CONCLUSIONS

1. One hundred and twenty blood serums from apparently healthy individuals were tested for agglutinins for sheep cells at 37 and 6 C. Of these, 98 (81.7%) were positive at 37 C. The titers varied from 1:4 to 1:128. All of the serums were positive for sheep cell agglutinins at 6 C. The titers were higher at 6 C than at 37 C, since the last varied from 1:64 to 1:1024.

2. The sheep cell agglutinins present in the human serums which acted at 37 C were absorbed when the serums were incubated with living bacteria of *Staphylococcus aureus*, *Brucella abortus*, and *Proteus OX19*, with living mature larvae of *Trichinella spiralis*, and adults of *Schistosoma mansoni*.

3. Sheep cell agglutinins were also absorbed when the serums were treated with well pulverized worm material from adult *Necator americanus*, *Ascaris lumbricoides*, and *Dirofilaria immitis*.

4. Absorption of sheep cell agglutinins by the living and dead organisms suggests that the agglutinins are closely related to infection and that they may develop following infection with any species of infectious agent.

5. It is suggested, therefore, that during infection the organisms secrete the A_2 isoagglutinogen-like substance, which immunizes the host. The antibodies formed can be detected as agglutinins for sheep cells.

6. In the blood serums from 25 out of 26 individuals (96.1%) infected with microfilariae of *Wuchereria bancrofti*, no agglutinins for sheep cells were detected. This suggests that in the serum from these individuals the substance being constantly secreted by the parasite neutralizes the antibody formed and that therefore no antibody, but antigen, may be detected.

THE SEROLOGICAL IDENTIFICATION OF CULTURES OF *CLOSTRIDIUM HISTOLYTICUM* AS GROUP III OF THE PROTEOLYTIC CLOSTRIDIA

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In a serological study of strains of *Clostridium sporogenes*, Mandia and Bruner[1] showed that identification of proteolytic clostridia was possible by means of antigenic formulas based on the reactions of the heat-labile antigens. Of the 25 *Cl. sporogenes* strains which they studied, 18 formed a homogenous serological group and was referred to as group I of the proteolytic clostridia. Three strains designated as *Cl. sporogenes* formed a second group with a strain of *Clostridium parabotulinum*. When a number of strains of *Cl. parabotulinum* and related organisms were included in the study, all reacted as members of the second group.[2] This group was then referred to as serological group II of the proteolytic clostridia.

A strain designated as *Cl. sporogenes* did not fall into either groups I or II, but showed, however, an agglutinative relationship to a strain of *Clostridium histolyticum*. Since the strain of *Cl. sporogenes* and the strain of *Cl. histolyticum* did not possess any of the 12 previously determined antigenic factors found in the organisms of groups I and II, they were considered as the third serological group observed for the proteolytic clostridia. It was felt that this third group might include other strains of the species *Cl. histolyticum*.

Information on the agglutination behavior of *Cl. histolyticum* is quite meager. It is limited almost to its agglutinative relationship to *Cl. sporogenes*. Smith[3] showed that a strain of *Cl. sporogenes* was antigenically almost identical with a rough variant of *Cl. histolyticum*. Mandia and Bruner[1] demonstrated a common heat-labile antigen between the two species. The agglutination of *Cl. histolyticum* itself can be summed up in the comment by Topley and Wilson[4] that agglutinating serums seem to act chiefly on homologous strains.

MATERIALS AND METHODS

Since *Cl. histolyticum* appears to be an organism found principally in gangrenous processes in man, cultures received for this study were from that source. Twelve cultures were received from the collection of Dr. L. D. Smith, who obtained them originally from Dr. John MacLennan. These cultures were H-3, H-8, H-9, H-12, H-13, H-14, H-16, H-17, H-24, H-27, H-31 and H-37. Strain CH-1, a laboratory stock culture, was included in the study. All cultures were characteristic of the species; that is, they were pathogenic, microaerophilic, spore-forming, proteolytic rods not fermenting any sugars.

The preparation of antigens and antiserums and the agglutinin absorption tests were carried out in the same manner as described for *Cl. sporogenes*.[1] In this study, however, a marked difference was noted in the amount of growth produced in the horse meat infusion broth by strains of *Cl. histolyticum*. Growth in 24-hour broth cultures was extremely heavy, requiring the dilution of the antigen 1 part to 2 parts of saline in order to obtain satisfactory agglutination tests.

Strains H-9, H-12, H-14, H-17 and CH-1 were nonmotile while strains H-3, H-8, H-13, H-16, H-24, H-31 and H-37 were motile. Motile organisms produced a heavy, sluggish spreading growth

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1. Mandia, J. W. and Bruner, D. W. 1951, J. Immunol. **66**: 497–505.
2. Mandia, J. W. 1951, J. Immunol. **67**: 49–55.
3. Smith, L. 1937, J. Bact. **34**: 409–417.
4. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of bacteriology and immunity, ed. 3, Baltimore, The Williams and Wilkins Co.

TABLE 1.—*Reactions of heat-labile antigens of strains of* Cl. histolyticum *antigens.*

Antiserums	Antigenic factors	Motile strains								Nonmotile strains				
		H-3	H-8	*H-13**	H-16	H-24	H-27	H-31	H-37	*H-9**	H-12	H-14	H-17	CH-1
H-13	13, 14	5000	5000	*5000*	5000	2000	2000	2000	2000	*2000*	2000	2000	2000	2000
H-9	13	200	500	*500*	100	200	200	100	500	*5000*	2000	2000	5000	5000
H-13 absorbed by H-9	14	500	500	*1000*	1000	1000	1000	500	1000	–	–	–	–	–
H-9 absorbed by H-13		–	–	–	–	–	–	–	–	–	–	–	–	–

* Selected type strain.
– Negative at dilution of 1:100.

in semisolid medium,[5] while the nonmotile organisms formed a dense growth confined to the line of inoculation. Antigens for the nonmotile strains of *Cl. histolyticum* were prepared as described for the nonmotile *Cl. sporogenes* strain, S-10, of group I.[1]

Antiserums were prepared for all of the strains except H-31 and H-37. When the 13 *Cl. histolyticum* strains were tested in these 11 antiserums, a homogenous group was formed. The *Cl. sporogenes* strain O-4 which reacted with *Cl. histolyticum* H-3[1] did not react with all of the antiserums of the group. It was considered, therefore, as not having the group antigen and was not included in this group. The agglutination of *Cl. histolyticum* was divided into two types depending upon the motility or nonmotility of the strain. Motile organisms produced a floccular agglutination when incubated for 1 hour at 50 C, while nonmotile strains produced large granular clumps only after 1½ to 3 hours of incubation.

RESULTS

When each of the 11 antiserums were absorbed by the cells of the motile strain H-13, no agglutinins remained for any of the motile or nonmotile strains. However, when they were absorbed by cells of the nonmotile strain H-9, a factor remained in the antiserums of the motile strains which reacted only with motile strains and not with nonmotile strains. This showed that both motile and nonmotile strains had a heat-labile antigen in common and that the motile strains had an additional heat-labile factor shared only by themselves.

Strain H-13 was selected as the type strain for the motile organisms and H-9 the type strain for the nonmotile organisms. The reactions of the heat-labile antigens of the strains of *Cl. histolyticum* in the antiserums and absorbed antiserums of the type strains are shown in table 1. The antigenic factor common to both the motile and nonmotile strains was assigned the numeral 13, being the 13th determined for cultures of proteolytic clostridia. This factor was present alone in the H-9 antiserum. When the H-9 antiserum was absorbed by the cells of strain H-13, all of the agglutinin factors for the group were removed. The H-13 antiserum absorbed by the cells of H-9 showed that a factor remained which reacted only with the motile strains. This factor was assigned the numeral 14.

Heat-stable antigens of H-13 and H-9 were prepared by boiling the 24-hour broth cultures for 2 hours. When these were tested in the antiserums of the group, a finely granular agglutination appeared upon standing at room temperature overnight. The antiserums, therefore, of both motile and nonmotile strains of *Cl. histolyticum* possessed agglutins for the heat-stable fraction. In similar antiserums of *Cl. sporogenes*,[1] only the nonmotile strains possessed agglutinins for the heat-stable antigen. When the motile H-13 antiserum was absorbed by the boiled H-13 cells, agglutinins for the heat-stable fraction were removed; the heat-labile (13 and 14) agglutinin factors remained. Also, when the nonmotile H-9 antiserum was absorbed by the boiled H-9 cells, the 13 factor remained which reacted only with the untreated cells.

Since the species of *Cl. histolyticum* is a member of the group of organisms

5. Mandia, J. W. and Bruner, D. W. 1950, J. Lab. & Clin. Med. **35**: 652–653.

TABLE 2.—*Proposed antigenic formulas and gradations of pathogenicity of* Cl. histolyticum.

Strains	Antigenic formula	Source	Pathogenicity following intramuscular injection of guinea pigs with 0.5 ml of 24-hour broth culture
H-3, H-16, H-37	Prot. *Cl.* III: 13, 14	Gas gangrene	Slight necrosis at the site of injection occurring within 24 hours. The necrotic area healed after 48 to 72 hours and the animal recovered.
H-8, H-13	Prot. *Cl.* III: 13, 14	Gas gangrene	Hemorrhagic liquefaction of the softer portion of the limb, spreading to the abdomen and followed by death within 48 hours after injection.
H-9, H-17, CH-1	Prot. *Cl.* III: 13		
H-24, H-27, H-31	Prot. *Cl.* III: 13, 14	Gas gangrene	Slight hemorrhagic liquefaction of the skin and muscle, with death occurring within 24 hours after injection.
H-12, H-14	Prot. *Cl.* III: 13		

known as the proteolytic clostridia, the heat-labile antigenic factors as determined (table 1) can be used to assign antigenic formulas to the 13 strains, following the scheme used for proteolytic clostridia of groups I and II. The antigenic factor 13, which is not found in organisms belonging to groups I and II, constitutes another group antigen. Therefore organisms demonstrating this factor form the third serological group of the proteolytic clostridia, or group III. The antigenic formulas for the strains of *Cl. histolyticum* studied are shown in table 2.

Pathogenicity

Besides demonstrating the heat-labile antigens, the 24-hour broth cultures prepared as described[1] also reflected the pathogenicity of the strains of *Cl. histolyticum*. When 0.5 ml of an untreated 24-hour whole broth culture of each of the 13 strains was injected intramuscularly into the thigh muscles of guinea pigs weighing from 450 to 600 g, 3 different gradations of pathogenicity were manifested. Weakly pathogenic strains H-3, H-16 and H-37 (table 2) demonstrated a slight necrosis of the site of injection occurring within 24 hours. The necrotic area usually began to heal after 48 to 72 hours and the animal recovered. Moderately pathogenic strains H-8, H-13, H-9, H-17 and CH-1 produced a hemorrhagic liquefaction of the softer portion of the limb, spreading to the abdomen and followed by death within 48 hours after injection. Very pathogenic strains H-24, H-27, H-31, H-12 and H-14 produced an apparent slight hemorrhagic liquefaction of the skin and muscle, with death occurring within 24 hours after injection.

The characteristic pathogenic reaction in guinea pigs injected with similar amounts of 24-hour broth cultures of *Cl. histolyticum*[6–8] follows the pattern shown by the moderately pathogenic organisms of this study. This variation in pathogenicity was not associated with any difference in antigenic make-up of the strains as determined in this study. However, the weakly pathogenic organisms (prot. *Cl.* III:13,14) gave a stronger reaction with the *Cl. sporogenes* strain O-4 than did the others which reacted only slightly or not at all. The antigenic factor common to *Cl. sporogenes* O-4 and the strains of *Cl. histolyticum* was not determined.

SUMMARY

Thirteen strains of *Clostridium histolyticum* formed a third serological group of the proteolytic clostridia. The pathogenicity of these strains varied. Some strains were very pathogenic and others were moderately pathogenic, characteristic of the species, or less pathogenic. Antigenic formulas were assigned to the strains studied.

6. Hall, I. C. 1922, J. Infect. Dis. **30**: 445–504.
7. Torrey, J. C. 1925, J. Infect. Dis. **36**: 517–524.
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EFFECT OF SELECTED CHEMICAL SUBSTANCES ON PHAGOCYTOSIS

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A consideration of the fundamental mechanisms of phagocytosis directed attention toward the effects of certain groups of compounds, namely the synthetic surface-active agents, on this phenomenon.

There are two chemical classes of surface-active agents, the nonionic and the ionic. Members of the ionic class of agents are, in turn, separated into two main divisions by the nature of the electrical charge assumed by the hydrophobic portion of the molecule upon ionization, namely, the anionic if negatively charged and the cationic when positively charged. An adequate discussion of compounds included in each of these categories is given by Schwartz and Perry.[1]

The development of synthetic surface-active agents has been directed primarily to practical industrial applications. Several recent symposiums[2,3] have reviewed the controversial theories of the state of dispersion of the synthetic surface active agents and other colloidal electrolytes. It is sufficient to indicate here that many surface active ions combine with or are adsorbed by proteins[4–8] and thus produce such diverse effects as protein precipitation and denaturation, inactivation of viruses, enzymes and toxins, splitting of natural complexes as plant virus nucleoproteins, hydrolysis of protein amide and peptide bonds, bacteriostasis, and hemolysis. In a general way, it has been shown by the interaction between pure proteins and synthetic surface active agents[9] that these phenomena are governed by mass relations between the reactants, by a relationship of surface active affinity to chemical structure, by pH and by ionic charge of the reactants. Likewise, they may combine in a chemical sense with other vital substances as lipids or carbohydrates.

Leukocytic response to surface-active agents has been investigated in relation to tissue toxicity of antiseptics, and a technique has been described for the concomitant study of the antiseptic's effect on phagocytosis of staphylococci, as well as for its bactericidal effect upon the same organism.[10] All data in these studies pertained to the concentrations of surface-active agents capable of stopping phagocytic activity. No attention was reserved for the interesting possibility that in other concentrations some of the surface-active compounds might stimulate phagocytic activity. This as-

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2. American Chemical Society. 1944, Symposium on the Effect of Surface Active Agents on Biological Systems.
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10. Welch, H. and Hunter, A. C. 1940, Am. J. Pub. Health, **30**: 129–137.

pect of the problem has been studied by Berry and associates[11] in an attempt to place the theoretical formulations of Fenn[12] on an experimental basis. They found that 5 out of a total of 52 surface-active agents tested in vitro increased both the number of bacteria ingested per neutrophile and also the total number of neutrophiles which phagocytized the test organism. Further, an increase in in vivo phagocytosis was noted when the most effective of these compounds, "triton" N-100, was injected intraperitoneally into mice. A similar treatment, however, failed to protect mice against experimentally induced infections with *Salmonella typhimurium*. These results were explained entirely on the basis of surface energy relationships in phagocytosis. No attention was given to the contributing role of chemical interaction between the surface-active agents and the cells involved.

METHODS

The present experimental series was initiated with two of the major types of surface-active compounds, that is, anionic and cationic substances. Although several so-called nonionic detergents were examined, the results are not reported because of uncertainty that they were truly nonionic. A previous publication from this laboratory[13] described the procedure for preparing the phagocytic test system, including white blood cell collection from donor animals and standardization of cell suspensions. Appropriate concentrations of the surface active agents, adjusted to pH 7.0, were introduced into the test systems placed in 10×70 mm tubes. This system usually contained:

(a) 0.3 ml of bacterial suspension (a heat-killed standardized suspension of a β-hemolytic streptococcus, New York 5)

(b) 0.2 ml of the substance to be tested for opsonic power (or serum or Locke solution for the control systems)

(c) 0.5 ml of the leukocyte suspension

The tubes, sealed with paraffin-dipped corks, were rotated gently end over end at 37 C for 15 minutes. Duplicate films on slides were made from the contents of each tube and stained with Wright's method. In each smear, at least 100 leukocytes were examined for the condition of the cell and its phagocytic activity. The system used for grading the activity was essentially that of Hamburger,[14] in which the percent of the phagocytes containing bacteria was determined.

RESULTS

Surface active-substances on phagocytosis.—The results of these studies are summarized in table 1. In general, it will be noted that the higher concentrations (10^{-3} to 10^{-4} g per ml) of both the anionic and cationic agents damaged the leukocytes. This effect was augmented when the systems did not contain serum. The protective action of serum is corroborated by Ponder,[15] who discovered that both the protein and lipoid components of plasma could inhibit red blood cell hemolysis by surface-active agents. Thus, he inferred that alkyl sulfates and probably other surface-active compounds could combine with the lecithin and cholesterol of the cell wall as well as with the protein to induce cytolysis. By this specific combination, then, rather than by their surface tension lowering properties, these compounds initiate lysis. It is felt that this point of view deserves emphasis.

Glassman[16] presents further evidence indicating that there may be a specific interaction between the surface-active agent and the cell surface. By comparing the behavior of nonionic and ionic compounds in concentrations giving equivalent surface tensions, ionic com-

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16. Glassman, H. N. 1947, Special Rept. #83, Surface Active Agents, Camp Detrick, Maryland.

TABLE 1.—*Action of some surface-active substances on guinea pig phagocytes.*

Classification of surface-active agent	Trade names	Type of substance	Concentration, g/ml	Surface tension in dynes*	Percent phagocytosis†			
					Presence of surface-active substances and		In absence of surface-active substance and	
					Serum	Locke's	Serum	Locke's
Anionic	Duponol	Fatty alcohol sulfate	10^{-3}	23.0	Ruptured	Ruptured	60–65	4–5
			10^{-4}	22.7	50–75	Ruptured	60–65	4–5
			10^{-5}	51.5	85–90	5	60–65	4–5
			10^{-6}	61.8	95	4–5	60–65	4–5
	Nacconal	Sodium alkyl aryl sulfonate	10^{-3}	24.5	Ruptured	Ruptured	65–70	5–10
			10^{-4}	42.4	85–90	Ruptured		
			10^{-5}	37.21	85	5		
			10^{-6}	62.1	70	5		
	Alconox	Said to contain polyalkylated amines, higher fatty alcohols, polyphosphates	10^{-3}	37.4	20	Atypical	65–70	5–10
			10^{-4}	40.2	45	1–2		
			10^{-5}	62.6	56	1–2		
			10^{-6}	63.2	60	1–2		
	Tergitol	Sodium salt of higher primary alkyl sulfate	10^{-3}	22.7	Atypical	Atypical	50–60	7
			10^{-4}	24.0	20–25	Atypical		
			10^{-5}	37.0	20	0		
			10^{-6}	62.5	35	0–2		
Cationic	Phemerol	Alkyl dimethyl benzyl-ammonium chloride	10^{-3}	24.6	Atypical	Atypical	60–65	4–5
			10^{-4}	28.9	20	10–15		
			10^{-5}	34.6	50	25–30		
			10^{-6}	54.1	55–60	20–25		
	Sapamine A	Diethyl aminoethyloleyl amide acetate	10^{-3}	21.0	Atypical	Ruptured	55–65	1–3
			10^{-4}	38.7	60–65	Ruptured		
			10^{-5}	63.4	70–75	Ruptured		
			10^{-6}		65–70	1–2		

* Surface tension of Locke's control = 63.2. These measurements were made by Dr. David English.
† Percent of phagocytes containing bacteria.

pounds are found to be markedly hemolytic as compared to solutions of nonionic compounds. The results in table 1 do not show a correlation between surface tension lowering and effects on the fragility and activity of phagocytes. This supports Ponder's and Glassman's contention that specific interactions with cell surface components are more significant than surface tension lowering in altering cell structure and function.

Lower concentrations of several test substances, including anionic "duponal" and "naccanol" and cationic "sapamine A," when used with serum enhanced the percent phagocytosis over that of the serum alone. Phemerol, a quaternary ammonium compound, was the only substance which gave any indication of being able to stimulate phagocytosis in the absence of serum. Increased phagocytic activity was not observed in the systems containing serum probably because of the inactivation of the compounds by the proteins and lipids of the serum. A group of 27 compounds, listed in table 2, chemically related to phemerol for the most part but lacking its surface tension depressing action, were supplied by Dr. Fleming of Parke, Davis and Co.

Nonsurface-active substances on phagocytosis.—This series of compounds was investigated for their effects on phagocytosis. The results, summarized in tables 3 and 4, were also verified by warm stage observations of treated and untreated cells by techniques reported by Merchant.[17] Table 4 indicates the results of ten of the compounds which stimulated phagocytic activity of white cells beyond that of untreated controls. It was hoped that one or more of these compounds could be employed in vivo as an artificial opsonin.

It was recognized that the use of such agents as those presented in table 4 in

17. Merchant, D. J. 1950, J. Infect. Dis. **87**: 275–284.

TABLE 2.—*Chemical nature of compounds studied for action on phagocytes and effect on phagocytosis.*

Compound no.	Chemical name	Effect in increasing phagocytosis in presence of: Serum	Locke's
1	β-Acetoxyethyl, dimethyl, carbethoxy methyl ammonium bromide	0	0
2	Allyl diethyl (2-hydroxyethyl) ammonium bromide 2-naphthalene carbamate ester	0	0
4	(2-hydroxytrimethylene) bis (triethylammonium iodide) carbanilate ester	0	0
5	Triethyl-α-naphthylmethyloxyethyl ammonium bromide	0	0
6	Ethyl (n). hexadecyl 2-ketomorpholinium bromide	0	0
7	β-benzhydryloxyethylmethylmorpholinium p-toluene-sulfonate	0	+
8	Dimethyl, 2-ketomorpholinium bromide	0	0
9	β-benzohydryloxyethyldimethyl-2-ethylhexylcarboxy-methylammonium chloride	0	0
10	β-benzohydryloxyethyltrimethylammonium bromide	0	+
11	β-benzohydryloxyethyldimethylcarbethoxymethylammonium chloride	0	0
12	β-benzohydryloxyethyldimethyl-γ-phenoxypropylammonium bromide	0	0
13	β-benzohydryloxyethyldimethyl-(n)-actylammonium bromide	0	0
14	β-benzohydryloxyethyldimethyl-(n)-amylammonium bromide	0	0
16	Carbamidomethylisoquinolinium chloride	+	+
18	β-hydroxyethyldimethyl-γ-phenoxypropylammonium bromide	+	+
19	β-benzohydryloxyethyldimethylpentadecylammonium bromide		
20	Spiro-(2-ketomorpholine-4,4′-morpholinium) bromide	0	+
21	Di(carbethoxymethyl)piperidinium bromide	0	+
22	Diethyl-2-ketomorpholinium bromide	0	0
23	Butenyl, dimethyl, carboethoxymethylammonium bromide	0	0
24	Methyl-6-hydroxyquinilinium-p-toluenesulfonate	0	0
25	Diethylmorpholinium p-toluenesulfonate	0	0
26	β-formyl-β,β-dimethylethyltrimethylammonium bromide	0	+
27	"Carbowax 1000 Monostearate"	0	0
28	Cetylpyridinium chloride	0	0
29	Cetyldimethylbenzylammonium chloride	0	0
30	Cetyltrimethylammonium bromide	+	+

parenteral therapy would be restricted by their toxicity. Tests performed in mice showed that four were sufficiently nontoxic to warrant trial in infected animals. Use of the four for protective action in mice infected intraperitoneally with type I pneumococcus demonstrated the effectiveness of two of the compounds, Spiro-(2-ketomorpholine-4,4′-morpholinium) bromide, and beta-formyl-beta, beta-dimethylethyltrimethyl ammonium bromide, hereafter to be referred to as no. 20 and no. 26, respectively.

Mice were inoculated intraperitoneally with 10^{-6} ml of type I pneumococcus and treated with various dosages of either no. 20 or no. 26. The survival time of these was compared with similarly inoculated animals which received 0.5 ml of 0.85% sodium chloride solution. The results, as seen in tables 5 and 6, reveal that all untreated controls died before the treated animals, including those receiving the smallest single daily dose of the drug. It was found that small doses (0.1 to 0.5 mg) administered several times daily were more effective in protecting the mice than a single large dose.

Effect of selected substances, no. 20 and no. 26, on experimental infections.—The results of trials of no. 26 in treating pneumococcus type I, 2 or 3 infections in mice indicated that the effectiveness of this compound was primarily limited to the type I pneumococcus strain employed although slight protection was afforded against the type 3 strain used. Also, this compound failed to protect mice against infections with a virulent *Proteus*, *Bacillus anthracis* and *Klebsiella pneumoniae*. Slight evidence of protection was evidenced against a β-hemolytic streptococcus, group C. It appears that the protective effect of no. 26 was quite specific, a most unusual finding.

Rats were inoculated intrabronchially with type I pneumococcus suspended in mucin in order to test the protective action of the compounds against pneumococcus pneumonia. The animals receiving 1 mg of no. 20 three times daily lived longer than the controls, 85 as compared to 49 hours, and eight of the 28 treated rats survived. None of 31 untreated rats survived. In the experiments in which no. 26 was used for therapy, the survival period for treated rats that ul-

timately died was 80 hours and that of untreated controls, 40 hours. Four of 20 treated rats survived while all of the 18 untreated controls died.

Mechanisms responsible for protective action of no. 20 and no. 26.—The protective effect of the compounds against the strain of type I pneumococcus employed in this work having thus been established, experiments were undertaken to further define the mechanism of this action. First, the bacteriostatic properties

TABLE 3.—*In vitro action of selected chemicals on guinea pig phagocytes. Substances which did not stimulate phagocytosis.*

Chemical no.	Number of experiments	Percent phagocytic activity and condition of cells					
		Concentration 10^{-3} g/ml		Concentration 10^{-4} g/ml		Concentration 10^{-5} g/ml	
		Serum	Locke's	Serum	Locke's	Serum	Locke's
1	4	35–39 N	0–5 N	43–45 N	0–5 N	55–59 N	0–5 N
2	4	33–35 N	5–10 N	35–40 N	0–3 N	55–60 N	0–5 N
4	4	20–25 V	0 D	35–40 V	0 V	48–50 N	0–2 90 N 10 V
5	4	28–30 N	0 SG–	30–35 N	0 50 N 50 S	40–43 N	0–2 N
6	4	0 D	0 D	44–46 90 N 10 D	0 D	50–58 N	0–5 N
8	4	32–35 90 N 10 D	0 D	52–64 N	0–2 90 N 10 D	60–65 N	0–7
9	4	0 S	0 S	26–29 N	0 S	50–55 N	2–5
11	4	0 D	0 D	20–26 80 N	0 D	30–35 97–98 N 2– 3 D	0 50 N 50 D
12	4	0 S	0 S	30–35 70 N 30 S	0 S	40–47 90 N 10 S	0 50 N 50 S
13	4	0 V	0 D	0 25 N 75 V	0 D	10–20 V	0 V
14	3	0 D	0–5 GR	10–20 V	0 D	40–50 N	0 V
19	3	0 D	0 GR	15–17 G–	0 D	20–30 G	0 D
22	3	11–12 N	0 S	32–36 N	0 N	40–50 N	0–1 N
23	3	14–15 N	0 N	30–36 90 N 10 S	0 50 N 50 S	30–50 N	0–2 N
24	3	9 N	0 D	12–15 N	0 N	25–30 N	0 N
25	3	0 D	0 D	0 D	0 D	10–12 V	0 D
27	3	12 75 N 25 D	0 D	15–25 95 N 5 D	0 50 N 50 D	35–40 N	0 95 N 5 D
28	3	0 D	0 D	8–10 V	0 D	20–27 90 N 10 V	0 V
29	3	0 D	0 D	5–10 50 N 50 D	0 D	20–30 75 N 25 V	0
Control series 16		Serum 55–65% N		Locke's 0–5% N			

D = phagocytes disrupted; G– = phagocytes lack granules; GR = phagocytes show abnormal arrangement; N = phagocytes normal in appearance; S = phagocytes shrunken; V = vesicles noted on some cells.

TABLE 4.—*In vitro action of selected chemicals on guinea pig phagocytes. Substances which stimulated phagocytosis.*

Chemical no.	Number of experiments	Percent phagocytic activity and condition of cells					
		Concentration 10^{-4} g/ml		Concentration 10^{-5} g/ml		Concentration 10^{-6} g/ml	
		Serum	Locke's	Serum	Locke's	Serum	Locke's
7	4	70–70 C	22–25 C	55–60 N	5–7 N	60–62 N	0–5 N
10	4	20–25 D	0 D	20–25 D	34–40 C	45–50 D	5–10 D
16	4	65–70 N	0–5 N	70–80 C	10–15 C	50–60 N	5–7 N
18	4	75–80 C	0–5 N	80–88 C	25–30 C	70–72 C	15–20 C
20	4	0 G	0 G	35–40 N	35–40 N	65–70 N	25–30 C
21	4	14 N	0 N	25–27 N	35–40 C	50–60 N	10–15 N
26	4	22 V	0 D	40–50 V	35–40 V	55–60 N	30–35 V
30	4	0 D	0 D	80–85 C	5–10 D	60–65 N	30–35 C
Control series 12		Serum 55–60% N		Locke's 0–5% N			

N = phagocytes normal in appearance; D = phagocytes disrupted; S = phagocytes shrunken; C = specially clear differentiation of stained cells; V = vesicles noted on some cells; G = phagocytes lack granules.

of no. 20 and no. 26 were determined for pneumococcus types I, 2 and 3, hemolytic streptococcus, *K. pneumoniae*, and *B. anthracis*. Concentrations of 10 mg per ml of medium of no. 20 were necessary to prevent the growth of all the test organisms. This concentration of no. 26 was not bacteriostatic except with very low inoculums of the test organisms. The requisite concentration of either drug for growth inhibition of these organisms was approximately 1000 times the concentration estimated to be present in a treated mouse.

A possible mechanism by which no. 20 and no. 26 might act in vivo would involve the metabolism of these drugs by the animal with the production of antibacterial substances having greater activity than shown by the compounds in vitro. To test this hypothesis, rats were injected intracardially with 4 mg of no. 20 or no. 26 in 2 ml of saline. The animals were sacrificed by bleeding at one hour, three hours, and 18 hours after inoculation. The serum samples obtained were tested for their antibacterial properties against type I pneumococcus and β-hemolytic streptococcus; no such action was detected. The action of breakdown products, if present, is very limited.

It has already been noted that no. 20 and no. 26 both increased phagocytosis of a killed suspension of hemolytic streptococcus, New York 5. Similar findings were made using pneumococcus type I suspensions.

The defense mechanism of the intact animals was next studied. First, the in vivo phagocytosis in mice as affected by these drugs was investigated. Each animal received intraperitoneally 0.5 mg of no. 20 or no. 26, or an equivalent volume of saline plus 10^{-3} ml of either type I pneumococcus or β-hemolytic streptococcus. The animals were sacrificed at intervals of 15 minutes, two hours, and four hours. The peritoneal cavity was rinsed with 2 ml of saline and duplicate films on slides made from the recovered fluid. One film of each pair was stained by Wright's method and the other with Gram's technique. The previously described procedure was used to measure phagocytosis. When possible, 100 leuko-

TABLE 5.—*The treatment of pneumococcus type I infections in mice with spiro (2-ketomorpholine 4,4'morpholinium) bromide (no. 20).*

Treatment	Approx. 10 LD_{50}		Approx. 100 LD_{50}		100 +LD_{50}	
	Survivors inoculated	Survival time	Survivors inoculated	Survival time	Survivors inoculated	Survival time
None	0/8	30 hr	0/8	24 hr	0/8	24 hr
No. 20 1.0 mg 1×/day	0/8	60–72 hr	0/8	50–70 hr	0/8	60 hr
None	0/20	28–35 hr.	0/20	24 hr.		
No. 20 0.1 mg 3×/day	2/20	60–72 hr.	0/20	45–55 hr.		
None	0/40	30 hr.	0/40	24 hr.	0/20	24 hr.
No. 20 0.5 mg 3×/day	35/40	120 hr.	0/40	72–80 hr.	0/20	40–50 hr.

cytes were examined, but frequently the limited number of cells on the slides resulted in an observation of but 50 cells. Slight evidence of intraperitoneal phagocytic activity was discovered on examination of these slides. Bacteria were on all of the slides from untreated animals. In the treated series although the organisms were easily demonstrated on hour and three hours, and the spleens removed. A sample of the blood and a portion of the finely ground spleens were plated in appropriate dilution onto blood agar. The plates were incubated, counted and the organism distribution charted as shown in tables 7 and 8. A pattern evident at one hour is well-defined at three. The organism concentra-

TABLE 6.—*The treatment of pneumococcus type I infections in mice with β-formyl-β-dimethylethyltrimethyl ammonium bromide (no. 26).*

Treatment	10 LD_{50}		100 LD_{50}		100 +LD_{50}	
	Survivors inoculated	Survival time	Survivors inoculated	Survival time	Survivors inoculated	Survival time
None	0/18	50– 60 hr.	0/18	40– 48 hr.	0/18	24– 30 hr.
No. 26 1.0 mg 2×/day	4/18	50– 96 hr.	0/18	70– 90 hr.	0/18	60 –80 hr.
None	0/10	36– 40 hr.	0/10	34– 40 hr.		
No. 26 0.1 mg 3×/day	4/10	90–120 hr.	0/10	90–136 hr.		
None	0/80	40– 55 hr.	0/80	40– 45 hr.	0/50	24– 30 hr.
No. 26 0.5 mg. 3×/day	57/80	90–140 hr.	19/80	70–120 hr.	0/50	60–120 hr.

the 15-minute specimens, by two hours a marked reduction of pneumococci was noted, and at four hours very few could be found.

This disappearance of the pneumococci in mice treated with no. 20 or no. 26 suggested an experiment to determine the in vivo fate of the test organism. Rats were inoculated intracardially with no. 20, no. 26, or an equivalent volume of saline plus type I pneumococcus. The animals were sacrificed by bleeding at 15 minutes, one tion in the blood of treated rats was significantly less than in that of untreated animals. At the same time, the concentration of pneumococci in the spleen was greater in the animals receiving either no. 20 or no. 26. These effects suggest an in vivo opsonic action in which phagocytosis by the fixed phagocytic cells of the spleen is markedly increased.

At this point in the program it was observed that cultures of pneumococcus type I growing in brain heart infusion broth containing 1 to 10 mg per ml of

TABLE 7.—*Pneumococci in blood and spleen of rats at intervals following intracardial inoculation with and without no. 20.*

Experiment	Treatment no.	15 minutes $\times 10^3$	1 hour $\times 10^3$	3 hours $\times 10^3$
		Blood		
1		400	50	100
	20	500	4	5
2		400,000	500	600
	20	500,000	50	50
3		650,000	600	30
	20	40	70	4
		Spleen		
1		400	60	70,000
	20	400	700	100,000
2		400,000	50	60
	20	400	1,000	200
3		6	90	100
	20	300	100,000	80,000

no. 26 produced a sediment in the bottom of the tube and a completely clear supernatant. This effect was noted to a limited extent with pneumococcus type 3 but not with one strain of type 2 nor any other organism yet tested. (See fig. 1.) The apparent specificity of this effect is quite striking. The effect is entirely devoid of bacteriostasis since plate counts of the resuspended sediment are equal to counts of control cultures without the drug.

The effect of no. 26 on the reaction between pneumococcus polysaccharides and their specific antiserums was tested. One mg of this drug per ml of polysaccharide solution greatly reduced the amount of precipitate formed with antiserum in a dilution of $1:10^3$ of pneumococcus I polysaccharide. This effect was not noted in higher ($1:10^2$) concentrations of polysaccharide, in which dilution reactions occurred in the control series. With pneumococcus type 3 polysaccharide, the volume of precipitate was reduced by no. 26, but precipitation in a dilution of $1:10^6$ of the polysaccharide still occurred in the presence of the drug. No effect of the drug on the reaction between pneumococcus type 2 polysaccharide and its antiserum was noted. The blocking effects of these substances are being further investigated with quantative techniques by Mr. Frederick Kull.

TABLE 8.—*Pneumococci in blood and spleen of rats at intervals following intracardial inoculation with and without no. 26.*

Experiment	Treatment no.	15 minutes $\times 10^3$	1 hour $\times 10^3$	3 hours $\times 10^3$
		Blood		
1		3	50	40,000
	26	5	30	5
2		80,000	100	400,000
	26	100,000	200	90
		Spleen		
1		20	20	5
	26	0.06	60	60,000
2		30	60	500
	26	100,000	300	50,000

It has been observed that the completely clear supernatant fluid of type I pneumococcus growing in the presence of no. 26 on occasions becomes turbid

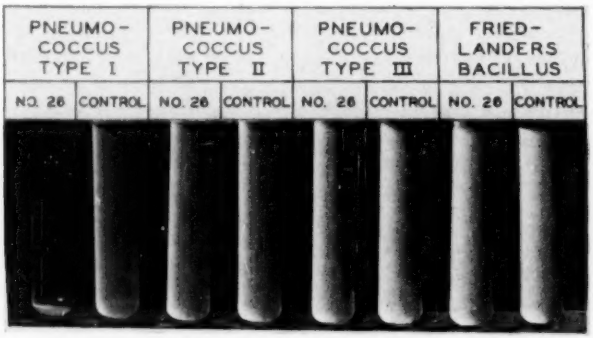

FIG. 1.—The effect of no. 26, 0.1% (β-formyl-β,β-dimethylethyltrimethyl-ammonium bromide), on growth in brain-heart infusion broth.

with further incubation. It has been surmised that the polysaccharide formed during growth ultimately combined with and inactivated the drug. This thought finds confirmation in the fact that 1.0 mg of SI added to 10 ml of medium with 1 mg of no. 26 prevents the sedimenting type of growth.

Another type of experiment which indicates a union between no. 26 and SI is the neutralization of the therapeutic effects of no. 26 by SI on pneumococcus type I vaccine. In these experiments, solutions of no. 26 were treated with a heavy vaccine of pneumococcus I or *K. pneumoniae* and then filtered through a Seitz pad. Another sample of the drug was treated with SI. Mice inoculated with pneumococcus type I intraperitoneally were then treated with saline, the drug alone or the "neutralized" drug, prepared as indicated. The percent survivors of 10 mice in each group was as follows: (a) No treatment—0%, (b) no. 26 alone—90%, (c) no. 26 treated with SI—10%, (d) no. 26 treated with pneumococcus I vaccine—0%, and (e) no. 26 treated with *K. pneumoniae* vaccine—50%. It thus appears that pneumococcus I vaccine or SI can neutralize the therapeutic effectiveness of no. 26 compound.

It seems that no. 26 simulates pneumococcus type I antibody in several ways. Obviously, the chemistry of the two is entirely different. However, the question has been raised as to whether or not the no. 26 molecule contains in a physical sense, a significant portion of the surface of the antibody molecule concerned with its reaction with SI. Work is in progress to further develop this consideration.

DISCUSSION

It was hoped that a study of the effect of various types of substances known to concentrate at surface interfaces and to lower surface tension would show differences in ability to stimulate phagocytosis. One of the cationic surface-active substances investigated, phemerol (alkyldimethylbenzyl ammonium chloride), did just this. However, another cationic substance, as well as anionic detergents, had no such effect in nontoxic concentrations. Furthermore, other substances, not surface-active, had similar effects. Thus it was not possible to support the hypothesis that surface tension lowering is a critical property of substances which function as opsonins.

Many of the substances examined were related to phemerol in that they possessed a tetra-ammonium structure. By no means, however, was this a cardinal characteristic insuring activity in increasing phagocytosis. It is well to avoid additional generalizations on the relation of chemical structure to the ability of a substance to increase phagocytosis, but rather deem it sufficient to emphasize the fact that relatively simple substances may increase phagocytosis. It should be kept in mind that such substances may act either on surface components of the phagocyte or on intracellular systems, as in the case with ascorbic acid. Increasing phagocytic activity is probably much more complicated than the simple alteration of the relative electrostatic charges or surface energies of parasite and phagocyte. Further study of this important phenomenon holds a challenge for those interested in the fundamental aspects of resistance. Also those with a healthy skepticism for the current trend of thinking only of the bacteriostatic drugs for the treatment of infectious diseases could well be on the alert for methods to increase the natural mechanisms of resistance by further investigation of the phenomenon of phagocytosis.

An entirely unexpected finding has come from these studies, namely, the

effect of two compounds, no. 20 and no. 26, in protecting mice against a strain of type I pneumococcus and a specific action in greatly altering the growth characteristics of this particular organism in liquid medium. The relation of structure of this substance to its effects on phagocytosis in vitro and in vivo, its immunological reactions, and its influence on growth characteristics as reported here are being studied further by Mr. Frederick Kull. Stimulated by the results obtained to date, the authors wish to emphasize the opportunities offered by studies of relatively simple compounds as an approach to the phenomenon of immunological specificity or specificity in general. Is it possible that these simple compounds simulate the distribution pattern of physical forces of the reactive groupings on the antibody macromolecules?

SUMMARY

A number of surface-active compounds, anionic and cationic, were studied in several concentrations for their effect on phagocytosis in the presence or absence of serum. The cationic quaternary ammonium compound phemerol attracted attention since it stimulated phagocytosis in the absence of serum. There seemed to be little relationship between surface tension lowering and ability to stimulate phagocytosis.

Thirty selected compounds, mainly not surface-active, but more or less structurally related to the quaternary ammonium nucleus of phemerol were studied. Two of these were shown to increase phagocytosis in vitro in the presence of serum, to increase phagocytosis in vivo and to protect mice and rats against several lethal doses of pneumococcus type I. The compounds were not significantly bacteriostatic.

One of these compounds has been found to specifically effect pneumococcus type I and to produce a sediment growth in broth. This effect, as well as the therapeutic effect in mice, is neutralized by SI. In turn, this compound interferes with SI-anti-SI reactions and to a slight degree, S3 and anti-S3, but not S2 and anti-S2 antigen-antibody reactions. Thus, it appears to mimic pneumococcus type I antibody in several significant ways.

ELECTROPHORESIS STUDIES OF LEUKOCYTES AND BACTERIA IN RELATION TO THE MECHANISMS OF PHAGOCYTOSIS

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Since the work of Rhumble[1] and Tait[2] and the theoretical formulations of Fenn[3] surface energy relationships of phagocytic cells have been considered important factors in deciding whether or not particles could be phagocytized. Unfortunately, there are no methods available for measuring interfacial tension between the surfaces of phagocytes and particles to be phagocytized. Electrophoresis techniques, however, can and have been used to investigate the possible role of alterations in surface charge of cells involved in phagocytosis. This paper reports the results of electrophoretic studies of bacteria and phagocytes in the presence of surface-active compounds in two major categories, anionic and cationic. Several so-called nonionic detergents were also investigated, but these data were not included due to uncertainty as to whether or not the substances were actually nonionic. In addition, several compounds are included which are not surface-active as far as lowering surface tension is concerned, but which have been shown to increase phagocytosis under appropriate conditions.

EXPERIMENTAL

The equipment and techniques for the electrophoretic measurements have been discussed at length by Abramson et al.[4] The electrophoresis apparatus used in the present series of investigations consisted of a microcell of the type described by Northrup and Kunitz.[5] It has two sealed-in platinum electrodes to which was attached an oscilloscope for reading directly the potential drop across the field. The cell was connected to nonpolarizing electrodes of Cu-$CuSO_4$ by very short rubber tubes. At first, plaster-of-Paris plugs were interposed to prevent migration of $CuSO_4$ from the electrode chamber into the cell. Later, a sintered glass boundary (medium grade) and a side arm controlled by a glass stopcock installed on the cell side of the membrane were built into the electrode vessels to enable the operator to flush out any $CuSO_4$ which diffused through the sintered glass prior to each run. Time formerly lost by the frequent replacing of fouled or cracked plaster-of-Paris plugs was thus saved.

Fresh human erythrocytes, suspended in the same buffer, were used early in these studies to determine a cell constant for changing observed velocities to mobilities in terms of microns per second per unit potential gradient. Abramson[6] has shown that the cataphoretic velocity of human erythrocytes is quite constant and equal to 1.19 to 1.35 μ per second per volt per cm when suspended in phosphate buffer.

Reliance on calibration with a biological material did not seem entirely satisfactory. Also, it was desirable to make frequent measurements of the potential gradient during an experiment. Hence, direct physical methods for measuring the potential gradient were sought. This can be done in two ways. Knowledge of the current flow and the resistance of the solution can be used to calculate the potential gradient. A second procedure involves measuring the actual potential between two platinum electrodes sealed into the cell. This value, along with knowledge of the distance between these electrodes, supplies the necessary data for determining the potential gradient. The latter method is the most direct and convenient, providing voltage is measured with a minimum of

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current flow so as to avoid the complications of polarization potentials. A very high resistance volt meter must then be used. One of the best meters for this purpose is the oscilloscope, having its amplifiers disconnected. Connections from the cell are made directly across the vertical plates of the oscilloscope with a connection of each plate to ground through a resistance of 5 megohms. Therefore, for each volt of potential across the cell, only 0.1 microampere flows between the two electrodes. The oscilloscope must be calibrated in volts per centimeter of deflection of the beam. This is easily done by employing a battery, the potential of which can be measured by the usual methods. In all work reported in this paper, the potential gradients were measured repeatedly during the course of an experiment by the oscilloscope method.

It is recognized that the electrophoretic mobilities as measured in the microscopic cell vary with the level in which the particle is floating. It is accepted practice to make measurements at levels 0.21 or 0.79 of the depth of the cell from the top. In this work, eight or nine different measurements were made on each particle. This data when plotted gave a curve; two values corresponding to the 0.21 and 0.79 levels were used in calculating mobilities. Also, similar curves were made with the polarity of the cell reversed.

The Tiselius moving boundary instrument was also used during the course of these studies. This apparatus is not ordinarily used for particles as large as leukocytes or bacteria. However, by increasing the viscosity of the buffer solution slightly with egg albumin or gum ghatti, good boundaries were obtained. The mobilities as calculated from Tiselius apparatus data agreed very closely with values obtained with the Northrup-Kunitz micro-cell.

Electrophoretic measurements were made on three types of particles: (a) β-hemolytic streptococcus (New York 5). The culture was grown for 18 hours in brain-heart infusion broth at 37 C. It was then concentrated and washed by repeated centrifugation and resuspension in 0.85% saline. The cells were heat-killed at 80 C for 30 minutes. Turbidity was standardized at 37% transmission on a photoelectric nephelometer (Lumetron), using distilled water as 100% transmission. A 1:5 dilution of this stock suspension was found satisfactory for electrophoresis measurements. (b) Pneumococcus type I. A five- to six-hour culture grown in brain-heart infusion broth at 37 C was used. The standardization followed the same procedure described for the streptococcus. (c) Guinea pig leukocytes. The method for obtaining leukocytes from guinea pigs in large quantities has been described in detail in a previous paper from this laboratory.[7] It was found that a 1% suspension of the leukocytes made from packed cells gave a satisfactory concentration for the electrophoretic measurements.

As has been shown by others,[8] the electrophoretic mobility of a particle depends upon both the organism and the suspending fluid in which the measurements are made. It was necessary in the course of these investigations to use four different suspending fluids. Therefore, the electrophoresis of all particles was measured in each of the solutions. The four fluids employed were: Locke's solution, pH 7.0; *M*/15 phosphate buffer, pH 7.0; normal guinea pig serum diluted 1:2 with phosphate buffer, pH 7.2; and immune guinea pig serum diluted 1:2 with phosphate buffer, pH 7.2.

Because of the importance assigned to energy relationships at cell surfaces in most explanations of phagocytosis, surface-active agents belonging to the anionic or cationic groups were employed to produce surface alterations. Although both surface tension and surface tension time drop measurements were made on many of the systems using the DuNuoy tensionmeter, it is recognized that such measurements are not entirely suited to the purpose. They do not measure the interfacial tensions between phagocyte and bacteria. Unfortunately, no method is available for making this crucial measurement. The effects of the various concentrations of the surface-active agents on phagocytic activity were measured by the traditional method of Hamburger[9] and have been reported.[10]

In addition to the anionic and cationic surface-active compounds, several other chemical materials, not surface-active but shown in earlier studies[11] to enhance phagocytic activity, were also introduced into electrophoretic systems for observation. One of these compounds possessed an action remarkably specific for pneumococci. Hence this organism was included in these studies.

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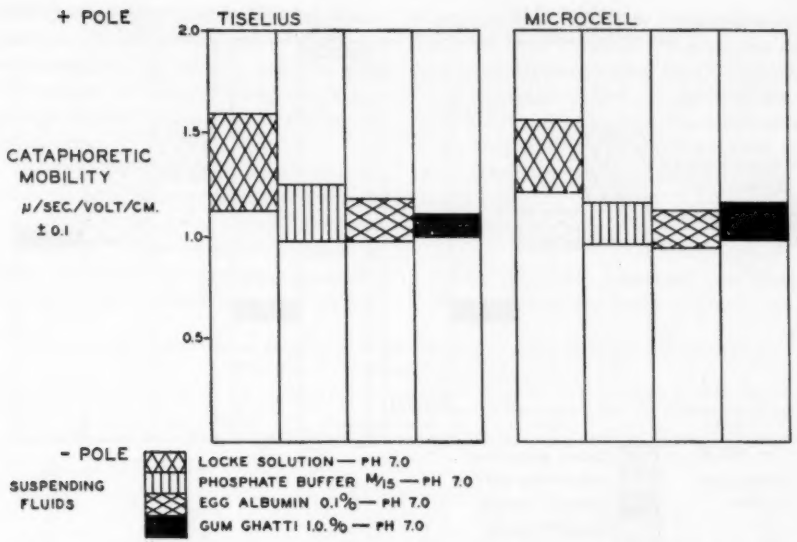

FIG. 1.—Comparison of cataphoretic mobilities of β-hemolytic streptococci, New York 5, in various suspending fluids using Tiselius and micro-cell techniques.

RESULTS

Measurement of the electrophoretic mobilities of bacteria and leukocytes by the two methods, in the micro-cell and by the Tiselius moving boundary technique, gave quite similar results (see fig. 1). Thus it is only necessary to discuss data obtained with the more practical apparatus, the micro-cell.

The ranges of measured mobilities of the bacteria or leukocytes investigated in each of several different suspending solutions are indicated in figure 2. In Locke's solution, pH 7.0, the particles moved more rapidly than in any other of the suspending mediums. The mobilities of particles suspended in $M/15$ phosphate buffer, pH 7.0, were somewhat slower than in Locke's solution. All particles, bacteria or leukocytes, moved slower when suspended in normal guinea pig serum diluted 1:2. The greatest change in mobility was observed in measurements of streptococci suspended in immune (antistreptococcus) guinea pig serum which had an agglutinating titer of 1:160. All particles observed, bacteria as well as phagocytes, when suspended in these fluids were electronegative. The correlation of the effects of normal and specific immune serums in decreasing the mobilities of the bacteria and increasing phagocytosis was made originally by Mudd et al.[12] This observation was based on the earlier findings of Northrop and DeKruif[13] that specific immune serum decreased the electrostatic charge of bacteria. Thus, the findings reported by the present authors extend these earlier observations by demonstrating that the leukocytes are also negatively charged and that their mobility is reduced by normal

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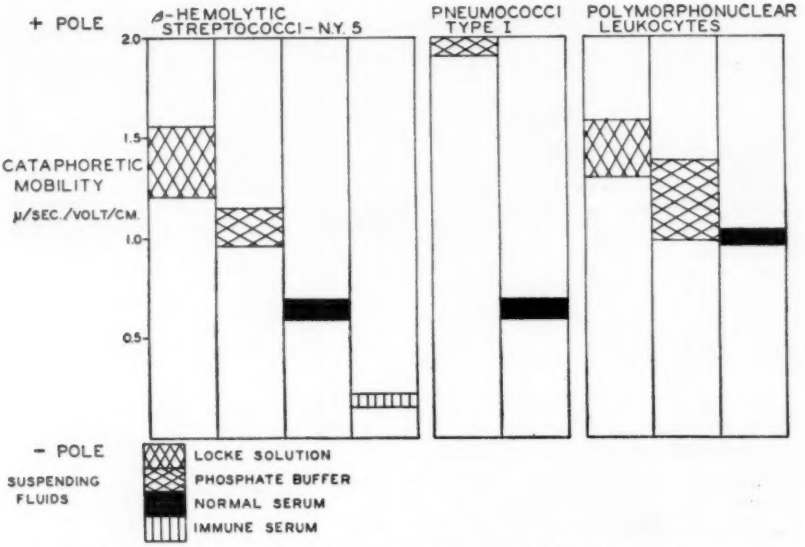

FIG. 2.—Comparison of suspending fluids on cataphoretic mobilities of streptococci, pneumococci, and guinea pig leukocytes.

homologous serum as compared to buffer solutions.

Anionic surface-active agents did not alter significantly the electrophoretic mobility of either bacteria or leukocytes, as illustrated by data in tables 1 and 2. The effects of Duponol are presented as representative of this group. Nacconol and Alkonox have given similar results. Cationic surface-active agents in high concentrations markedly slow the mobilities or even reverse the charge on bacteria in certain suspending fluids. Both Sapamine-A and Phemerol had this effect. These findings agree with the published data of Dyar and Ordal.[14] Normal serum as a suspending medium, however, prevented the reversal of charge, probably due to the com-

14. Dyar, M. T. and Ordal, E. J. 1946, J. Bact. **51**: 149–167.

TABLE 1.—*Mean cataphoretic mobility* of guinea pig polymorphonuclear leukocytes as affected by various types of substances and suspended in several fluids.*

Concentration of substance	Locke solution				Phosphate buffer				Normal guinea pig serum			
	A	B	C	D	A	B	C	D	A	B	C	D
10^{-1}												
10^{-2}						0.8						
10^{-3}					1.2	0.9			1.0		0.8	
10^{-4}	0.5	1.5			1.3	0.9			1.0		0.7	
10^{-5}	0.7	1.6			1.3	1.0			1.1		0.8	
10^{-6}		1.6			1.4	1.1			1.0	0.7	0.8	
10^{-7}		1.5			1.5	1.2			1.0		0.9	
10^{-8}	1.5			1.4	1.3	1.2		1.4	1.0		1.0	1.1
Control		1.4				1.2				1.0		

A = anionic agent—Duponol.
B = cationic agent—Phemerol.
C = quaternary ammonium compound—no. 20.
D = quaternary ammonium compound—no. 26.
* Mobility expressed in μ/sec/volt/cm. Values given represent the mean of data collected in at least three separate runs.

bining effect of proteins with these compounds. The data obtained with Phemerol presented in table 1 indicate that the cationic surface-active agents do not conspicuously affect the mobility of leukocytes.

Among the compounds studied for their effect on the mobilities of bacteria and leukocytes, two, though not surface-active, were selected because of their ability to stimulate phagocytic activity.[11] These compounds, quaternary ammonium salts, were Spiro-2-keto-morpholine-4,4′-morpholinium bromide, and β-formyl-β, β-dimethylethyltrimethylammonium bromide, to be indicated hereafter as numbers 20 and 26, respectively. The cataphoretic mobilities of streptococci in the presence of no. 20 and no. 26 are given in table 2. They had practically no effect. Pneumococci, alone of the several organisms observed,[11] were stimulated by compound no. 26 to form a sedimenting growth leaving a clear supernatant. Cataphoretic studies revealed that concentrations of 10^{-4} to 10^{-6} g per ml of this substance gave a mobility of 1.5 as compared to a control measurement of pneumococci suspended in phosphate buffer alone, of 2.2, or in normal serum, of 0.6. Thus, no. 26 had a greater effect on the mobility of pneumococcus than on streptococcus. A few measurements made on leukocytes in the presence of various concentrations of no. 26 and no. 20 are found in table 1. Leukocytes presented difficulties in the measurements of electrophoretic mobilities because of their tendency to settle in the cataphoretic cell.

CONCLUSIONS

From the data presented the most striking observation deals with the unusual situation that electronegatively charged leukocytes are attracted to and phagocytized by electronegatively charged bacteria. This presents a most unusual physical situation, particularly since the surface areas of the two types of particles are not greatly different. Also, it appears from the electrophoretic data presented in this paper and the phagocytic studies of the preceding paper that the electrostatic charge of bacteria or leukocytes as currently measured does not supply a satisfactory index as to the expected phagocytic activity in a system. Studies designed to reveal differences in electrostatic charge on parts of the leukocyte surface might give more significant findings. It seems quite probable, if not necessary, that the portion of the leukocyte surface which comes into contact with the elec-

TABLE 2.—*Mean cataphoretic mobility* of β-hemolytic streptococci as affected by various types of substances and suspended in several fluids.*

Concentration of substance	Locke solution				Phosphate buffer				Normal guinea pig serum				Immune guinea pig serum			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
10^{-1}		−0.9														
10^{-2}		−0.4				0.1										
10^{-3}	1.5	−0.2			1.1	0.9	1.0	1.5	0.6	0.5	0.5	0.7	0.2			0.2
10^{-4}	1.4	1.6			1.0	1.1	1.5	1.4	0.6	0.6	0.6	0.8	0.2			0.2
10^{-5}	1.2	1.5			1.0	2.2	1.3	1.6	0.6	0.6	0.6	0.8	0.2			0.2
10^{-6}	1.3	1.5			1.0	1.8	1.2	1.6	0.6	0.7	0.5	0.9	0.2			0.2
10^{-7}	1.3	1.5			1.0	1.2	1.4	1.7	0.6	0.5	0.6	0.7	0.2			0.2
10^{-8}	1.4	1.5			1.0	1.0	1.0	1.0	0.6	0.6	0.6	0.6	0.2			0.2
Control		1.5				1.0				0.6				0.2		

Values expressed—cataphoretic mobility in μ/sec/volt/cm.
A = anionic agent—Duponol.
B = cationic agent—Phemerol.
C = quaternary ammonium compound—no. 20.
D = quaternary ammonium compound—no. 26.
* Mobility expressed in μ/sec/volt/cm. Values given represent the mean of data collected in at least three separate runs

tronegatively charged bacteria should carry a zero charge, if not a positive one. The challenge remains to supply adequate data to support such a hypothesis. On the other hand, it is possible that too much attention has already been directed to surface tension and electrostatic charge, yet the crucial steps in the phagocytic process may not be fundamentally physical.

SUMMARY

Several modifications of the technique for measuring the electrophoresis of particles in a micro-cell have been made, including an electrode wash-out system and measurement of the potential gradient with an oscilloscope. Measurements of particles in the micro-cell and by the Tiselius technique gave similar results.

The mobilities of both bacteria and leukocytes measured in various suspending fluids were usually electronegative. In high concentrations of cationic detergents the charge of bacteria was decreased and even reversed. These substances had very little effect on the mobility of leukocytes. Mobilities of bacteria were greatest in Locke's solutions and somewhat less in phosphate buffer. They were slowed and stabilized to the greatest degree in immune serum.

LABORATORY STUDIES ON HISTOPLASMOSIS

I. THE EFFECT OF HUMIDITY AND TEMPERATURE ON THE GROWTH OF *HISTOPLASMA CAPSULATUM*

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Emmons[1] first reported the isolation of *Histoplasma capsulatum* from soil in 1949. He also reported that this organism would grow in the laboratory on sterilized soil with a high humus content. The organism sporulated freely when the soil was moistened and contained dung and humus. Recently[2] he reported the isolation of *H. capsulatum* from a total of 6 soil samples out of more than 400 tested and also demonstrated the presence of typical tuberculate spores in soil. Thus, it appears that *H. capsulatum* has a free-living saprophytic existence in soil. The conditions necessary for its growth in nature, however, have not been determined.

In an effort to determine the conditions necessary for the growth of *H. capsulatum*, experiments were conducted in which the fungus was grown on bark and soil at controlled temperatures and humidities in closed chambers. In addition, experiments were conducted with *Sporotrichum schenckii* and *Blastomyces dermatitidis*.

MATERIALS AND METHODS

Varying humidities were obtained by adding measured amounts of sodium chloride or sulfuric acid to distilled water. Data concerning vapor pressure lowering with sodium chloride were obtained from the *International Critical Tables*,[3] data for use of sulfuric acid from Parkes.[4] These methods of lowering vapor pressure appear to be relatively accurate as confirmed by the studies of Sweetman,[5] Buxton et al,[6] and Griffiths.[7]

In the experiments varying amounts of sodium chloride or sulfuric acid were added to measured quantities of distilled water in flasks, which were then autoclaved. Approximately 300 ml of each concentration of the sterile sodium chloride or sulfuric acid solution were placed in sterile jars. The 100% relative humidity jars contained only distilled water. The lids were tightly secured after inoculation.

Wide-mouth quart jars with screw-capped lids were used as chambers for the experiments. Inside the jars small petri dishes were taped to the top of Coplin staining dishes so that the fluid in the jars was at least 2 inches below the small petri dishes. The substrate to be inoculated (bark or soil) was placed in the small petri dishes. The jars were autoclaved before use.

The substrate which was placed in the small petri dishes in the jars consisted of several types:

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5. Sweetman, H. L. 1933, Studies of chemical control of relative humidity in closed spaces. Ecology, **14**: 40–45.
6. Buxton, P. A. and Mellanby, K. 1934, The measurement and control of humidity. Bull. Entomolog. Res., London, **25**: 171–175.
7. Griffiths, E. 1925, The measurement of humidity in closed spaces. Dept. of Scientific and Industrial Res., Food Invest. Bd. Spec. Rep. No. 8, 1–54.

1. Soil—surface soil gathered from the University grounds.
   a. Used as collected after autoclaving for 1 hour at 122 C and 18 pounds pressure without drying or addition of water.
   b. Wet—approximately 1 ml distilled water was added to each 6 g of soil used.
   c. Dried—consisted of soil which was heated in a gas drying oven for 3 days at temperatures of 110 to 130 C. During the third day the temperature was raised to 170 to 180 C for 1 hour.
2. Bark from live elm trees (*Ulmus americana* or *Ulmus fulva*) collected locally and used in small pieces (½×2 inches).
   a. Wet—the bark was floated in distilled water for ½ hour before being transferred to the petri dishes.
   b. Dried—the bark was dried in the same manner as the soil.

Growth was observed in the experimental chambers for periods of 6 to 8 weeks, after which the experiment was terminated and tests for viability of the organisms were performed. These tests were performed in two ways: (1) subculturing to appropriate mediums with observation for characteristic growth and the production of typical spores, and (2) placing the unopened jars at room temperature and observing for the appearance of typical growth, which was then subcultured.

The inoculum consisted of 1- to 3-month-old potato dextrose agar cultures of *H. capsulatum* (strain 629 from a human case), or a 6-week-old culture of *B. dermatitidis* (strain P48), or a 6-day-old culture of *S. schenckii* (Conant strain). A loopful of mycelium was scraped from the surface growth avoiding as far as possible inclusion of any medium. This loopful then was planted in a central, well marked location on the soil or bark which was to be inoculated.

EXPERIMENTS AND RESULTS

Twenty-two experiments were performed involving different temperature and humidity conditions for the growth of *H. capsulatum*. Six experiments were formed with *B. dermatitidis* and six with *S. schenckii*.

The results of all of the experiments are summarized in table 1, which shows the effect of different temperatures and varying humidities on the growth of the fungi. It is evident from the study of table 1 that *H. capsulatum* did not grow in any of the experiments at less

TABLE 1.—*Effect of humidity and temperature on the growth of* H. capsulatum, S. schenckii, *and* B. dermatitidis.

Organism	Temperature	Medium	Growth at designated percent relative humidity				
			100	98	97	96	93
H. capsulatum	5 C	Soil*	None	None†			
	10 C	Soil*	None	None			
	20–25 C	Dried bark	Fair	None	None	None†	None†‡
		Dried soil	Poor	None		None	None
		Wet soil	Good				
		Wet soil	Good				
		Soil*	Poor	None		None†	None†
		Soil*	Poor	None†		None†	None†
	30 C	Wet bark	Good§				
		Wet soil	Good				
		Wet soil	Good				
	40 C	Wet bark	None†§				
		Wet soil	None				
		Wet soil	None				
	50 C	Wet bark	None§				
		Wet soil	None				
		Wet soil	None				
S. schenckii	28–30 C	Dried bark	Good	Fair			Poor‖
B. dermatitidis	20–25 C	Wet bark	Good	None		None	None¶

Viability tests were performed at the end of the experiment on each jar. Unless otherwise noted, all viability tests were negative. Results of experiments employing less than 93 % relative humidity are recorded below.

* These soil samples were used as collected after autoclaving without drying or addition of water.

† Viable at the end of the experiment.

‡ 90%—none, 80%—none, 76%—none.

§ 90%—none, 76%—none, 64%—none.

‖ 87%—none, 80%—none, 76%—none.

¶ 83%—none, 67%—none, 57%—none.

than 100% relative humidity. In fact, this humidity dependence appeared to be so critical that the organism did not grow when the humidity was lowered by as little as 2%. This finding was true for both methods of lowering humidity and also was true for *B. dermatitidis*. On the other hand, *S. schenckii* was not nearly as critical in its humidity demands since growth was evident down through the 93% relative humidity but was absent from the 87%. These results with *S. schenckii* confirm earlier experiments reported by Brown et al.[8]

An interesting observation derived from table 1 is that *H. capulatum* has a relatively narrow temperature range for growth. No growth was observed at temperatures of 5 or 10 C nor at temperatures of 40 or 50 C. *H. capsulatum* grew at temperatures of from 20 to 30 C. Large fluffy white colonies which were quite similar to the growth seen on artificial laboratory mediums were observed on the bark. On the other hand, the growth in soil was considerably more difficult to observe, but on occasions the growth was quite evident and appeared similar to that seen on bark.

Another observation evident from table 1 is that the organism grew better on bark or soil which was wet prior to inoculation. If the soil was thoroughly dried, growth was usually meager and rather slow in appearing. When wet substrates were used the organisms grew faster and colonies were larger. In fact, definite growth first was observed at 1 week on the wet soil or bark but was delayed until 3 weeks on the dried soil or bark. Growth on the soil neither moistened nor dried was observed at 2 weeks. On the other hand, in certain unpublished experiments it was found that if the soil was wet excessively (4 ml or more distilled water added to each 6 g of soil), no visible growth occurred.

Also seen in table 1 are the results of tests for viability of the spores at the end of the observation period of 6 to 8 weeks.

Soil was not too satisfactory for the demonstration of viability as the inoculum disappeared into the soil and it was difficult to be certain that subcultures were made from the area in which the original inoculum had been placed. At 5 C one subculture was found to grow, which indicated that the spores had not been killed by standing at this temperature for 6 to 8 weeks. At 20 to 25 C a number of positive subcultures were obtained from experiments at different humidities, and it was found that the organism was not harmed by standing at this temperature even though no visible growth had been observed.

At 40 C one of 6 jars was found positive by culture, whereas at 50 C none of 6 jars was found viable after the observation period of 6 to 8 weeks.

DISCUSSION

The experiments reported in this paper serve to outline some of the conditions necessary for the growth of *H. capsulatum* under artificial conditions. Particularly striking were the humidity requirements of the organism. Growth occurred only at 100% relative humdity.

Besides humidity, temperature limited the growth of *H. capsulatum*. No growth was observed at 5 or 10 C nor at 40 or 50 C, whereas satisfactory growth occurred between 20 and 30 C.

It should be noted that the experiments conducted were done at constant temperatures and constant relative humidities in closed chambers. It is

8. Brown, R., Weintroub, D. and Simpson, M. W. 1947, Sporotrichosis infection on mines of the Witwatersrand. A symposium. The Transvaal Chamber of Mines, Johannesburg, South Africa, pp. 16–20.

difficult to make direct application of these experiments to conditions as they exist in nature. However, our knowledge of the humidity and temperature requirements of these microorganisms is so fragmentary that these experiments seem worth reporting.

SUMMARY

Several experiments were performed involving different temperature and humidity conditions for the growth of fungi on elm bark and soil. Most of the experiments were performed with *Histoplasma capsulatum*, although *Blastomyces dermatitidis* and *Sporotrichum schenckii* were utilized in similar experiments.

H. capsulatum did not grow in any of the experiments at less than 100% relative humidity. This finding was true for both methods of lowering humidity and was true also for *B. dermatitidis*. *S. schenckii* was not nearly as critical in its humidity demands since growth was evident down through the 93% relative humidity but was absent from the 87%.

H. capsulatum was dependent upon temperatures between 20 to 30 C for growth. No growth was observed at temperatures of 5 or 10 C nor at temperatures of 40 or 50 C.

H. capsulatum grew better on bark or soil which was wet prior to inoculation. When dried soil or bark was used the growth did not appear for 3 weeks, whereas it was evident at 1 week on the wet soil or bark.

Spores of *H. capsulatum* were found to remain viable after 6 to 8 weeks at 5 C. At 20 to 25 C a number of positive subcultures were obtained from experiments at different humidities. At 40 C one of 6 jars was found positive by culture, whereas at 50 C none of 6 jars was found viable after the observation period of 6 to 8 weeks.

RELATIVE EFFICIENCY OF DIFFERENT CULTURE MEDIUMS IN ISOLATION OF CERTAIN MEMBERS OF THE SALMONELLA GROUP

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The isolation of enteric pathogens from fecal material has presented many difficulties. That no one method or medium is perfectly satisfactory is quite apparent from the numbers that have been proposed and the successive modifications throughout the years. The relative efficacy of various methods and mediums for isolating salmonella species has been discussed by many workers. Some of the more recent evaluations are to be found in the following references: Hynes (1942), Knox et al (1942), Mollov et al (1943), Galton and Quan (1944), Neter and Clark (1944), Greifinger and Silberstein (1944), Felsenfeld (1945), Broh-Kahn (1946), and Galton and Hardy (1948). Workers have used different selections of mediums satisfactory to them and often modified existing mediums so that none of these studies are particularly comparable. It may be concluded from the vast amount of experience in this field that certain enrichment mediums are superior to direct culture for isolation of the salmonellas and that of the various selective and differential mediums in use, no one medium will allow satisfactory isolation of all species or strains. In general, the greater the number of methods and mediums employed, the greater the success in isolation.

During the course of an investigation of the pathogenicity of certain salmonellas for man (McCullough and Eisele, 1951), an array of culture mediums was used. Nearly 500 individual feedings of salmonella organisms were given, resulting in 96 cases of clinical illness. While the study employed only a few species and strains, repeated isolations were made from known cases and carriers, and the total number of isolates was considerable. Moreover, since known species and strains were fed repeatedly and standardized techniques were employed throughout, many of the variables usually present in an evaluation study of culture mediums were minimized. These data are being presented for what value they may have to other workers doing or contemplating similar studies. It cannot be assumed that the results necessarily reflect those which might be obtained with the entire gamut of salmonella species.

MATERIALS AND METHODS

Three strains each of *Salmonella meleagridis* and *Salmonella anatum*, one strain each of *Salmonella newport*, *Salmonella derby*, and *Salmonella bareilly*, and four strains of *Salmonella pullorum* were employed in the study. All of the cultures had been isolated from market samples of spray-dried whole egg by the Bureau of Agricultural and Industrial Chemistry, United States Department of Agriculture, with the exception of one strain of *S. pullorum* obtained from a case of human illness (Gernez-Rieux et al, 1949). The various strains were fed in graded doses to healthy human volunteers. Prior to feeding, the stools of each subject were repeatedly examined to determine the absence of salmonella species.

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After feeding, stool specimens were obtained three times a week for the first two weeks and once a week thereafter until the subjects ceased shedding organisms. At least three consecutive weekly negative cultures were obtained on each subject. In the first part of the work the men were followed for a much longer period until it became apparent that three consecutive weekly specimens were usually sufficient to establish negativity. For the purpose of this report, all fecal specimens obtained from subjects after feeding through the period including the last positive specimen obtained by any method were regarded as presumptive positive specimens.

Fecal specimens were obtained by inserting the gloved finger well within the rectum and having the subject bear down. Usually a gross fecal specimen was obtained. We have found this method to be far superior to the usual rectal swab method in obtaining fecal material.

The following culture methods were employed. Fecal specimens were streaked directly on the surface of SS (Salmonella-Shigella) medium (Difco) and BS (bismuth sulphite agar) medium (Difco) and at the same time were inoculated into two enrichment broths, TBG (tetrathionate broth (Difco) containing brilliant green 1:100,000 and SF (Selenite F) medium (BBL). These broths were incubated at 37 C for 18 hours. Each broth was then subcultured by streaking on the surface of SS agar medium, BS agar medium, EMB agar medium (Difco), and MAC (MacConkey) agar medium (Difco). In the earlier part of the work, DCLS (desoxycholate citrate lactose sucrose) agar medium (BBL) was also employed. With the exception of the bismuth sulphite plates, all plates were incubated for 24 hours at 37 C and examined. The bismuth sulphite plates were held for at least 48 hours. Colonies suspected of being salmonella were subjected to spot agglutination, the usual biochemical tests, and finally were typed according to the Kauffmann-White schema. With plates presenting suspicious colonies, three or four colonies were selected for tests and the plates preserved until the status of these cultures had been determined. If none of them proved to be salmonella, opportunity was still present to re-examine the plates.

RESULTS

Table 1 presents the comparative results of the direct culture and enrichment culture methods for all species other than *S. pullorum*. An inspection of the table reveals, as one might expect, some variation in results from strain to strain. Of the 1216 specimens presumed to contain salmonellas, 1,028, or 84.5%, were proved positive by culture. Direct culture on SS agar medium yielded slightly better recovery than on bismuth sulphite agar medium. Following each enrichment broth, total recovery of positive cultures was from 3 to 4 times that obtained with either direct culture method. There were 17 instances in which specimens were positive on one or both direct culture plates while the enrichment broths were negative.

Table 2 presents a comparative tabulation of the results obtained with the 2

TABLE 1.—*Percent recovery of certain salmonella strains from presumed positive fecal specimens by direct culture and by enrichment broths.*

Organism	Total number specimens	Number positive specimens by culture	Percent positive	Direct culture*		Enrichment broths		Total percent recovery	
				BS	SS	TBG	SF	Direct culture	Enrichment broths
S. meleagridis, strain I	298	256	85.9	24.2	25.3	81.5	71.4	30.6	85.5
S. meleagridis, strain II	232	199	85.7	17.4	15.2	71.9	68.5	24.0	83.6
S. meleagridis, strain III	85	79	92.9	17.6	24.7	82.3	84.7	30.5	90.5
S. anatum, strain I	131	110	83.9	14.9	22.0	79.3	66.4	26.7	82.4
S. anatum, strain II	188	147	78.1	12.4	20.5	68.0	64.3	24.3	75.5
S. anatum, strain III	112	91	81.2	22.9	36.6	72.3	69.6	43.1	80.3
S. newport	80	69	86.2	21.0	22.3	78.7	70.0	28.9	85.0
S. derby	56	45	80.3	13.4	11.5	66.0	62.5	15.3	80.3
S. bareilly	34	32	94.1	17.6	20.5	79.4	82.3	20.5	94.1
Total	1216	1028	84.5	18.6	22.3	75.7	69.8	28.0	83.1

* Direct culture results for *S. meleagridis*, strain I, were calculated on 284 specimens; for *S. meleagridis*, strain II on 229; for *S. anatum*, strain I, on 127; for *S. anatum*, strain II, on 185; for *S. anatum*, strain III, on 109; for *S. newport* on 76; and for *S. derby* on 52.

TBG—Tetrathionate broth (Difco) containing brilliant green 1:100,000.
SF—Selenite F broth (BBL).
BS—Bismuth sulphite agar (Difco).
SS—SS agar (Difco).

TABLE 2.—*Percent of total salmonella isolations obtained with two enrichment broths subcultured on four different mediums.*

Organism	Total specimens positive by culture	Total percent positive		Percent of specimens positive							
				TBG subcultured to				SF subcultured to			
		TBG	SF	BS	SS	EMB	MAC	BS	SS	EMB	MAC
S. meleagridis strain I	256	94.9	83.2	89.0	92.5	88.2	86.3	66.7	75.0	62.8	63.2
S. meleagridis strain II	199	84.4	79.8	70.3	78.3	69.8	66.3	61.3	67.3	55.7	55.7
S. meleagridis strain III	79	88.6	91.1	81.0	78.4	74.6	73.4	56.9	82.2	67.0	60.7
S. anatum strain I	110	94.5	79.0	87.2	88.1	83.6	81.8	71.8	72.7	54.5	55.4
S. anatum strain II	147	87.0	82.3	76.8	80.2	75.5	71.4	55.1	70.7	56.4	56.4
S. anatum strain III	91	89.0	85.7	78.0	84.6	80.2	72.5	58.2	80.2	64.8	60.4
S. newport	69	91.3	81.1	68.1	85.5	68.1	73.9	46.3	76.8	47.8	56.5
S. derby	45	82.2	77.7	66.6	60.0	57.7	46.6	51.1	57.7	46.6	33.3
S. bareilly	32	84.3	87.5	65.6	78.1	50.0	56.2	50.0	78.1	62.5	62.5
Total	1028	89.5	82.5	78.7	83.4	76.7	74.2	60.5	73.1	58.4	57.7

TBG—Tetrathionate broth (Difco) containing brilliant green 1:100,000.
SF—Selenite F broth (BBL).
BS—Bismuth sulphite agar (Difco).
SS—SS agar (Difco).
EMB—EMB agar (Difco).
MAC—MacConkey agar (Difco).

enrichment broths followed by subculture to 4 different mediums. Here again species and strain differences are readily apparent. Subculture of either enrichment broth to SS agar medium gave results superior to any of the other mediums. The over-all superiority of TBG broth is evident although relative strain differences exist. There was better recovery from this broth on each of the 4 mediums than that obtained with any medium following Selenite F broth. EMB agar medium gave results comparable to those with MacConkey medium following either enrichment broth.

These results show that employment of more than one medium yields a higher total recovery from either enrichment broth. It is of interest that the employment of EMB and MacConkey mediums combined in the entire study yielded isolations from only 32 specimens which were not otherwise proved positive.

DCLS medium was used in the earlier part of the work for subculture of both enrichment broths. *S. meleagridis*, strain I, and *S. anatum*, strain I, were the only strains then employed. The medium was used on 250 presumptively positive specimens, yielding from 51% recovery from Selenite F broth to 64% from TBG broth—results inferior to those with any of the other mediums. In no instance was an isolation made only on DCLS medium. The medium was then dropped from the study. From the results reported by other workers with this medium, it is probable that with some of the other strains it might have yielded more satisfactory results.

Four strains of *S. pullorum* were employed, one of these having been obtained from a case of human illness. The results with all 4 strains were similar. Of 32 specimens presumed as positive, *S. pullorum* was isolated from 29. Twenty-five of the specimens were positive from Selenite F broth, while 19 were positive from TBG. Direct culture on bismuth sulphite agar yielded 7 positive, on SS agar, 9. In one instance direct culture on SS agar gave the only isolation from that specimen. EMB and MacConkey mediums yielded 5 and 6 isolations from both enrichment broths;

SS agar 16 from TBG and 22 from Selenite F; BS agar 18 from TBG and 20 from Selenite F.

DISCUSSION

The positive fecal specimens forming the basis of this report were derived from groups of men receiving graded dosages of the respective strains. They represent subjects yielding a positive specimen on one occasion only as well as those with well established carrier states lasting several months, and those actually exhibiting clinical illness of mild to severe degree. Such an array of specimens probably furnishes a truer average sampling of the diverse conditions affecting successful isolation of salmonellas than a series derived entirely from cases or established carriers.

The relatively high percent of positive isolations in this study is of interest. Subject to the limitations of the mediums and technique employed in establishing persistent negativity of a subject's stools, it reflects the degree of constancy with which salmonellas may be found in the stools during the shedding state and the relatively abrupt termination of this state as compared to the typhoid carrier.

The results confirm the findings of some other workers in regard to the usefulness of some of the mediums employed in detecting the species represented here, in particular the results of Mushin (1948). She found that tetrathionate broth subcultured to SS agar gave superior results in isolation of *S. derby* from the stool in some 87 instances.

The isolation of *S. pullorum* from fecal specimens presents greater difficulty than the other species mentioned. Bushnell and Porter (1945) decided that there was no suitable medium for this purpose, but of those available, desoxycholate citrate agar, bismuth sulphite agar, and SS agar were most useful and tetrathionate broth as an enrichment medium was of value. In the small number of isolations reported here, the use of SS and bismuth sulphite agar mediums following one of the enrichment broths gave the best results, but it is considered that these results are far from those which might be desired.

SUMMARY

Data showing the relative efficiency of a variety of medium in detecting certain strains of *Salmonella* in presumptively positive fecal specimens is presented. The species of *Salmonella* employed were: *S. meleagridis*, *S. anatum*, *S. newport*, *S. bareilly*, *S. derby*, and *S. pullorum*. The mediums used for direct culture were SS agar and bismuth sulphite agar and for enrichment, Selenite F and tetrathionate containing brilliant green 1:100,000. Each enrichment medium was subcultured to bismuth sulphite agar, SS agar, EMB agar, and MacConkey agar. Both enrichment mediums yielded results greatly superior to those obtained on direct culture. The modified tetrathionate broth was superior to Selenite F. SS agar yielded superior results as a medium for subculture of either enrichment broth. The enrichment mediums subcultured to SS agar and bismuth sulphite agar yielded the most recoveries of *S. pullorum*.

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CHEMOTHERAPY OF EXPERIMENTAL CLOSTRIDIAL INFECTIONS

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Recent events have increased the interest in the treatment of clostridial infections. During World War II a considerable amount of information was accumulated on the efficacy of sulfonamides (McKee et al, 1943; Hac and Hubert, 1944; Hac, 1944a; Langley and Winkelstein, 1945; Ryan et al, 1946) and of penicillin (McKee et al, 1943; Hac, 1944b; Macfarlane, 1945) in the treatment of experimental and clinical gas gangrene.

With the advent of the newer antibiotics (Duggar, 1948; Ehrlich et al, 1947; Hobby et al, 1950) it was felt desirable to compare them with penicillin, which had shown encouraging results in earlier work.

A number of investigators have evaluated the effectiveness of aureomycin against experimental *Clostridium perfringens* infections (Altemeier et al, 1950; Sandusky 1950), but only one paper dealing with the experimental evaluation of aureomycin, terramycin and penicillin against *Clostridium septicum* and *Clostridium tetani* infections has come to our attention (Bliss et al, 1950).

IN VITRO TESTS

Methods

The tube dilution method of Dornbush and Pelcak (1948) was employed, using thioglycollate broth (N.I.H. formula) as the diluent and for the test culture. All cultures were grown 24 hours in thioglycollate broth and diluted 1:100 in the same medium. Five-tenths ml of a serial dilution of the antibiotic was placed in 10×75 mm Pyrex tubes and 1.5 ml of the diluted culture were added. The test was read after 18 hours of incubation at 37 C. The result was recorded as the lowest concentration in micrograms per ml required to inhibit development of visible turbidity.

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The *Clostridium* cultures tested were *tetani* Tulloch A, *histolyticum* H7C, *perfringens* BP6K, *septicum* VSB, *feseri* 169-10, *novyi* N-21B, *botulinum A* Hall, *botulinum B* Okara, *bifermentans* S-4 and *hemolyticum* ACC-29867. All except *Cl. hemolyticum* grew well in thioglycollate broth; this culture grew so poorly that a dilution of 1:10 was employed as inoculum in the tests.

The antibiotics employed were the potassium salt of penicillin G, aureomycin buffered with sodium glycinate to an approximate pH of 8.3, terramycin and chloramphenicol. All the chemicals were diluted just prior to use. Streptomycin was not included because Altemeier et al (1947) had shown in vivo and Robinson et al (1944) had shown in vitro that it was ineffective against the clostridia. The work of Bliss et al (1950) substantiated these findings.

Results

The results of these experiments are summarized in table 1. Aureomycin, penicillin G and terramycin activity was, in general, of the same order of magnitude. In most cases less than 0.2 μg per ml of these antibiotics inhibited growth; only *Cl. bifermentans* and *Cl. botulinum B* required more than 0.63 μg for bacteriostasis. Chloramphenicol was much less effective than the other three.

With this background of experience on the effectiveness of the antibiotics against the clostridia we felt that it would be desirable to evaluate them in vivo. Experience during World War II indicated that in clostridial myositis thorough surgical debridement was of utmost importance no matter which method of chemo- or serotherapy was used (Dowdy et al, 1944; Ulio and Marshall, 1945). For preliminary work at least, we believed that muscle trauma should be reduced to a minimum and that dirt or other foreign material should be excluded except in a few instances where calcium chloride solu-

TABLE 1.—*In vitro sensitivity of clostridia.*

Species	μg/ml				Ratio for bacteriostasis			
	Aureomycin	Terramycin	Penicillin	Chloramphenicol	Aureomycin	Terramycin	Penicillin	Chloramphenicol
Cl. bifermentans	2.5	>40.0	5.0	10.0	1	>16	2	4
Cl. botulinum B	1.25	20.0	1.25	10.0	1	16	1	8
Cl. tetani	0.16	0.63	0.02	2.5	8	32	1	125
Cl. histolyticum	0.04	0.32	0.0025	2.5	16	128	1	1000
Cl. botulinum A	0.02	0.16	0.16	2.5	1	8	8	125
Cl. feseri	0.02	0.16	0.02	2.5	1	8	1	125
Cl. perfringens, type A	0.02	0.16	0.08	2.5	1	8	4	125
Cl. septicum	0.02	0.08	0.005	2.5	4	16	1	500
Cl. hemolyticum	0.01	0.63	0.0025	0.63	4	250	1	250
Cl. perfringens, type D	0.01	0.16	0.08	5.0	1	16	8	500
C. novyi	0.005	0.08	0.0025	1.25	2	32	1	600

tion was used as a menstruum for spore suspensions when broth cultures were found to be unsatisfactory as infecting inoculums.

CHEMOTHERAPEUTIC TESTS

Methods

Cl. septicum VSB, *Cl. perfringens* BP6K, *Cl. histolyticum* H7C, *Cl. novyi* N-21B, *Cl. bifermentans* S-4, *Cl. tetani* Tulloch A and *Cl. feseri* 169-10 were used for the in vivo evaluation of the antibiotics. All cultures except *Cl. feseri* were tested in Rockland strain albino mice weighing approximately 20 g. Infection was made by injection into the thigh muscle of 0.1 ml of whole culture, washed whole culture resuspended in 2.5% calcium chloride, or a suspension of spores in 2.5% calcium chloride except for *Cl. tetani*, in which infection was subcutaneous at the base of the tail. The particular type of inoculum is specified in table 2, which summarizes the results of the in vivo tests. Cultures were grown in thioglycollate broth because little or no toxin was produced in this medium. Cultures used for infection were tested to be sure that soluble toxin was absent. *Cl. feseri* was tested in guinea pigs because it was not lethal to mice. Two-tenths ml of a suspension of spores in 2.5% calcium chloride was injected intramuscularly as an infecting dose. Therapy was begun one hour after infection. In most instances the drugs were administered intraperitoneally since pilot experiments with *Cl. tetani*, *Cl. septicum*, *Cl. perfringens*, *Cl. histolyticum* and *Cl. novyi* indicated that subcutaneous administration was much less effective.

Results

Cl. septicum proved very sensitive to aureomycin, terramycin and penicillin. When the antibiotics were administered subcutaneously at the rate of 2 mg/kg of body weight in one daily dose for two days, aureomycin and penicillin protected 100% and terramycin 75% of the mice. Two 1 mg/kg doses of aureomycin given intraperitoneally on successive days gave 100% protection.

One 2 mg/kg dose administered daily for 5 days by the intraperitoneal route provided 100% survival of *Cl. perfringens*-infected mice whether aureomycin, terramycin or penicillin was the antibiotic used. However, the penicillin-treated mice all showed large, crippling, palpable masses at the site of inoculation for about 10 days after infection. Gross evidence of residual lesions upon autopsy was noted only in the penicillin-treated group.

Aureomycin was somewhat more effective in combatting infections with *Cl. histolyticum* than was terramycin, and both were much more effective than penicillin. Two and five-tenths mg of aureomycin per kg protected 95% and 1 mg/kg protected 60% of the mice, while 5 mg of terramycin per kg protected 70% and 2.5 mg/kg protected 65%. Ten mg of penicillin per kg protected only 20% of the mice.

Cl. novyi, on the other hand, responded more readily to terramycin than to penicillin or aureomycin. Ten mg of aureomycin per kg gave only 30% protection, and the same amount of penicillin gave about 70% protection.

Aureomycin and terramycin were much superior to penicillin for treat-

TABLE 2.—*In vivo activity of antibiotics against clostridia.*

Antibiotic	mg/kg	Route of treatment	Number of doses	Number of tests	Survivors	Percent survival	Avg. hrs. survival
					Total		
			Cl. septicum				
Aureomycin	10	s.c.	2	2	17/17	100	
Aureomycin	2	s.c.	2	1	11/12	92	75
Terramycin	10	s.c.	2	1	12/12	100	
Terramycin	2	s.c.	2	1	9/12	75	78
Penicillin	10	s.c.	2	1	12/12	100	
Penicillin	2	s.c.	2	1	12/12	100	
Aureomycin	10	i.p.	2	1	10/10	100	
Aureomycin	5	i.p.	2	1	10/10	100	
Aureomycin	2	i.p.	2	1	10/10	100	
Aureomycin	1	i.p.	2	1	10/10	100	
Controls				4	0/27	0	18
Infecting dose: 0.01 ml spores in calcium chloride or culture i.m. = 0.96×10^8 cells in culture.							
			Cl. perfringens				
Aureomycin	10	i.p.	5	3	22/22	100	
Aureomycin	2	i.p.	5	1	10/10	100	
Terramycin	10	i.p.	5	1	10/10	100	
Terramycin	2	i.p.	5	1	10/10	100	
Penicillin	10	i.p.	5	1	10/10*	100	
Penicillin	2	i.p.	5	1	10/10†	100	
Controls				3	5/22	23	26
Infecting dose: 0.1 ml thioglycollate broth culture i.m. = 54.5×10^4 cells.							
			Cl. histolyticum				
Aureomycin	10	i.p.	5	2	20/20	100	
Aureomycin	5	i.p.	5	2	19/20	95	18
Aureomycin	2.5	i.p.	5	2	19/20	95	24
Aureomycin	1	i.p.	5	2	12/20	60	60
Terramycin	10	i.p.	5	2	18/20	90	22
Terramycin	5	i.p.	5	2	14/20	70	32
Terramycin	2.5	i.p.	5	2	13/20	65	31
Penicillin	10	i.p.	5	2	4/20	20	31
Penicillin	5	i.p.	5	2	6/20	30	39
Penicillin	2.5	i.p.	5	2	2/20	10	38
Controls				2	0/20	0	20
Infecting dose; 0.02 ml thioglycollate broth culture i.m. = 1.76×10^8 cells.							
			Cl. novyi				
Aureomycin	10	i.p.	5	2	6/20	30	46
Aureomycin	5	i.p.	5	2	5/20	25	45
Aureomycin	2.5	i.p.	5	2	0/20	0	42
Terramycin	10	i.p.	5	2	19/20	95	24
Terramycin	5	i.p.	5	2	20/20	100	
Terramycin	2.5	i.p.	5	2	15/20	75	58
Penicillin	10	i.p.	5	2	13/20	65	40
Penicillin	5 †	i.p.	5	2	15/20	75	41
Penicillin	2.5	i.p.	5	2	12/20	60	38
Controls				2	0/20	0	22
Infecting dose: 0.005 ml spores in calcium chloride i.m.							
			Cl. bifermentans				
Aureomycin	20	i.p.	2	1	12/12	100	
Aureomycin	10	i.p.	daily for 3 days	1	12/12	100	
Terramycin	20	i.p.	daily for 3 days	1	12/12	100	
Terramycin	10	i.p.	daily for 3 days	1	12/12	100	
Penicillin	20	i.p.	daily for 3 days	1	4/12	33	48
Penicillin	10	i.p.	daily for 3 days	1	2/10	20	36
Controls				1	0/12	0	23
Infecting dose: 0.01 ml thioglycollate broth culture i.m. = 4.75×10^8 cells.							
			Cl. tetani				
Aureomycin	20	i.p.	5	2	34/34	100	
Aureomycin	10	i.p.	5	2	10/14	72	58
Controls				2	8/34	23	56
Infecting dose: 0.001 ml spores in calcium chloride s.c.							
			Cl. feseri				
Aureomycin	10	oral	5	1	2/3	67	24
Aureomycin	5	oral	5	1	3/3	100	
Aureomycin	2.5	oral	5	1	2/3	67	96
Aureomycin	5	s.c.	5	2	19/20	95	96
Controls				3	5/23	22	38
Infecting dose: 0.0002 ml spores in calcium chloride i.m.							

i.m. = intramuscular.
i.p. = intraperitoneal.
s.c. = subcutaneous.
* Large crippling, palpable lesions persisting for 4 days. Other groups' negative.
† Large, crippling, palpable lesions persisting for 10 days.

TABLE 3.—*Summary of in vivo sensitivity of clostridia.*

Species	Mg drug/kg body weight required for 90–100% protection			Dosage		
	Aureomycin	Terramycin	Penicillin	Number of doses per day	Number of days	Route
Cl. tetani	20			1	5	i.p.
Cl. bifermenians	10	10	>20	2	3	i.p.
Cl. novyi	>10	5	>10	1	5	i.p.
Cl. feseri	5			1	5	oral
Cl. feseri	5			1	5	s.c.
Cl. histolyticum	2.5	10	>10	1	5	i.p.
Cl. perfringens	2	2	2	1	5	i.p.
Cl. septicum	1	2	2	1	2	i.p.

i.p. =intraperitoneal.
s.c. =subcutaneous.

ment of *Cl. bifermentans* infection. Two daily doses of 10 mg/kg continued for three days protected 100% of the mice when the first two antibiotics were used, but 20 mg of penicillin per kg protected only 33%.

Five doses of 20 mg of aureomycin per kg, one dose daily, were necessary to protect 100% of the mice against *Cl. tetani*. The other antibiotics were not tested.

Guinea pigs of the Hartley strain, weighing 225 to 250 g, were infected with *Cl. feseri* and treated orally with daily doses of aureomycin for 5 days. Five mg per kg protected all the animals, 2.5 mg/kg was slightly less effective and 10 mg/kg appeared to be slightly toxic. Guinea pigs are unique in that they do not tolerate aureomycin well and for this reason are not good experimental animals for use in tests with this antibiotic.

DISCUSSION

The correlation between in vitro and in vivo results in this series of tests was fairly good although *Cl. novyi* was very sensitive in vitro but quite resistant to all three antibiotics in vivo. *Cl. hemolyticum*, the etiological agent of icterohemoglobinuria in cattle, was not tested in vivo. *Cl. tetani* and *Cl. feseri* were tested before terramycin was available to us, so comparative tests were not done with these two organisms. In general, the in vivo results suggest that any of the three antibiotics should be effective against clostridial infections provided that therapy is initiated soon enough and that, in the case of the "gas gangrene" group, surgical debridement would be adequate and early.

SUMMARY

Ten species of clostridia proved to be sensitive to aureomycin, terramycin and penicillin in vitro in low concentrations. One hundred and twenty-five to 1000 times as much chloramphenicol was required for bacteriostasis. In vivo tests indicated that the first two antibiotics were effective in preventing death from experimental infections with any of the 7 species of clostridia tested. Penicillin was less satisfactory in infections with *Clostridium bifermentans*, *Clostridium novyi* and *Clostridium histolyticum*.

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BACTERIOLOGIC STUDIES OF EXPERIMENTAL SALMONELLA INFECTIONS IN CHICKS

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Bacteria of the genus *Salmonella* infect man and animals with the production of disorders which range from trivial to fulminating in their manifestations. Such infections, though frequent and world wide in their distribution, are not adequately controlled by current preventive measures or available chemotherapeutic agents. Consequently, the relative importance of salmonellosis as a public health problem is increasing.

In this country human salmonellosis is predominantly an enteric infection with a nonfatal outcome. In attempting to study the disease experimentally it would be highly desirable to be able to work with an infectious process of comparable clinicopathologic course. In the laboratory, however, attempts to produce experimental infections through deliberate exposure to cultured bacteria are often surprisingly unsuccessful despite the fact that an extremely wide range of animal hosts has been found to harbor these infections in nature and although spontaneously occurring salmonellosis frequently produces epizootics in colonies of laboratory animals. For various practical considerations the mouse has been chosen for most laboratory investigations of salmonella infections to date. The principal studies of pathogenesis and experimental epidemiology, work on the genetic and nutritional bases for resistance, and the great majority of laboratory therapeutic trials have all been performed with white mice, but they are by no means ideal for some experimental purposes. Inoculation and treatment by gavage, though feasible, are tedious because of the small esophagus and stomach. The difficulty of regularly obtaining blood and feces for culture from such a small animal renders bacteriologic follow-up uncertain. It is not an easy matter to mark a large number of mice so that individual identification is possible. More important, their susceptibility to different types and strains of salmonella varies markedly. With a few types, notably those which are natural pathogens in rodents, the introduction of a few bacilli will often produce in mice an acute disease with fatal outcome. With many other types, relatively large numbers of viable organisms when introduced in saline or broth suspensions by any of several routes are well tolerated unless the dose is so high as to induce death from toxemia. For such reasons in most laboratories attempting to evaluate the effects of vaccination or chemotherapy in experimental salmonella infections, it has become customary to adjust conditions so that a rapidly fatal outcome may be expected among inoculated but otherwise untreated mice in the control groups, thus permitting a quick reading

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of tests in terms of numbers of survivors without further bacteriologic check. In order to accomplish this, intraperitoneal inoculation, usually with large doses of viable cells or with mucin as an adjuvant, is ordinarily employed. Death which results in a day or two from overwhelming infection, with septicemia and possibly toxemia, is not always desirable, however. It precludes, for example, a genuine treatment experiment in which evidence of established infection is obtained before therapy is begun.

In searching for a better experimental animal our attention was directed to domestic fowl, particularly chickens. Chickens are natural hosts to salmonella—not only to *Salmonella pullorum* and *Salmonella gallinarum*, but to most of the salmonella types commonly found in man. As laboratory animals they have the possible disadvantages of requiring special feed and equipment for their maintenance, of growing large and resistant to infection unless they are used when very young, and of being birds rather than mammals and thus possibly not as comparable to human beings as other experimental animals. However, newly hatched chicks cost less on the average than weanling mice and are available almost everywhere. Inoculation by any of several commonly used routes, excepting possibly the intravenous route, is relatively simple, and specimens of feces for culture are readily obtained by introducing a swab into the cloaca. Heart punctures are easily performed and well tolerated. Serially numbered wing bands make permanent identification of individual birds convenient. Numerous groups of veterinary workers have used young or adult chickens, as well as turkeys and ducks, in the study of avian salmonellosis. The oral route of inoculation has been frequently employed, since this is considered to be a principal natural portal of entry. Evidence of infection has been derived in some instances from bacteriologic examination of the intestinal discharges taken during life and in other experiments from cultures of tissues at autopsy or on the basis of fatality. In nearly all investigations to date the inoculum of organisms has been considerably in excess of 10^6 viables, and it has been administered in single doses or repeated over several days.

In a few preliminary experiments we obtained evidence that very young chicks could be regularly infected with even small numbers of salmonella commonly pathogenic for man when the organisms were administered to the birds via the oral route, with the production of disease which like its human counterpart varied from inapparent to severe but was easily detectable by bacteriologic means over a period of weeks. Subsequent extensive use of chicks in this laboratory over a two-year period has adequately shown their utility for the investigation of many phases of salmonellosis. Because the experimental use of young chickens introduces problems not ordinarily encountered in laboratories for medical research and because techniques developed in this laboratory differ in important respects from those reported from other institutions where chicks have been employed, a rather full description of materials and methods is given here together with experimental data to describe: (1) the infectivity of various strains of salmonella for chicks of differing ages and breeds; (2) the nature of the infection produced, including duration, fatality, and bacteriologic findings associated with the disease.

MATERIALS AND METHODS

Chicks and their care.—Newly hatched chicks obtained from an intelligently managed local hatchery were transferred directly from the hatching trays to new shipping cartons and de-

livered to the laboratory where they were held in a warm room until used. Except as otherwise noted, all chicks were of the New Hampshire Red (NHR) breed hatched from eggs supplied by a single producer (CL). This strain was selected because of the size and vigor of the chicks and their year-round availability.

One minor difficulty encountered in our use of hatchery chicks was the occasional finding of animals harboring salmonella. Although the eggs were from a producer who had reported no "pullorum reactors" for several years, *S. pullorum* was isolated from these chicks on several occasions. Because of the anticipated possibility of confusion in the experimental results, use of this bacterial species was deliberately avoided in our experimental inoculations. Also, in two instances there were recovered salmonella of another type which had not been employed experimentally. It was impossible to trace the sources of contamination but it is surmised that the chicks were infected at the hatchery where, of necessity, eggs from more than one flock were often set in the same large forced-draft incubator. This type of spread has been previously demonstrated (Buxton and Gordon, 1947). There were very few experiments invalidated by such unwanted infections, partly because the bacteriologic methods employed insured their prompt discovery and partly because, under the environmental conditions afforded, the pullorum infections showed little tendency to spread among the animals of the experimental groups. Furthermore, it was found that experimental infections could be readily superimposed on the naturally occurring ones (which, in our experience, were seldom fatal), with results which could be usefully interpreted for some purposes.

Work with experimentally infected chicks in limited space in our institutional animal house demanded the use of special equipment and rigorous sanitary techniques in order to maintain satisfactory isolation among groups of chicks and to prevent the spread of infection to other varieties of animals in the same building. The methods employed here have been highly successful in these respects. Multiple-tier commercial brooders could not be used because contaminated material passed readily from top to bottom in the models tested. To circumvent this difficulty single, electrically-operated, commercial brooder units of the contact heat type (capacity: 50 to 60 chicks up to 3 weeks of age) were mounted in the centers of large metal pans which extended beyond the extreme dimensions of the brooders by several inches on all sides and were then placed one over the other on a frame of galvanized iron pipe which was suspended from the ceiling. Six units of this type were employed. For some experiments, however, it was desirable to use as many as 12 small groups of chicks, and no method of subdividing the commercial units was considered to give adequate isolation. An experimental type of brooder was devised, therefore, to provide additional isolated units in the small space available. White enamelware pans 10 inches wide, 16 inches long, and 4½ inches deep, such as are used in restaurant steam-tables, were fitted with: (a) covers of ½ inch mesh hardware cloth, (b) small feed and water troughs with hinged wire restrainers and hooks to support them at the proper height, and (c) a thin layer of bagasse placed on the bottom and covered with several thicknesses of newspaper. One of these pans would adequately house 8 chicks up to 15 days of age. Three such pans were accommodated and kept at approximately 90 F in the specially constructed brooder. This consisted of a base made in the form of a flat pan 24 inches wide, 43 inches long, and ½ inch deep, on which was mounted a peak-roofed "hover"; both were constructed of galvanized steel. The hover (18 inches wide, 42 inches long, and 15 inches high at the center) contained a 15-watt light bulb and a thermostatically controlled 100-watt bar heating element; it had a hinged flap in front which could be opened for convenience in inspecting the chicks. Four brooders of this type were also supported on a pipe frame suspended from the ceiling.

All brooders used for salmonella studies were housed in a single room, from which personnel unaffiliated with the project were excluded. The walls, ceiling, and other exposed surfaces were painted with an emulsion of DDT at two to three month intervals to discourage possible insect vectors. Persons engaged in cleaning cages or other operations likely to contaminate the floor donned rubber boots which were disinfected after each use and never taken out of the room. The entire floor was mopped with a cresol disinfectant before and after each manipulation of cages or birds.

An average population of nearly 100 infected chicks produced a large volume of infectious waste matter which had to be collected and removed from the room frequently. A 30-gallon covered garbage can was kept in the room to receive all contaminated wastes such as droppings, dead chicks and unused feed; it was also used as a lethal chamber for sacrificing chicks with chloroform at the termination of each experiment. When the large brooders were to be cleaned, the trays containing animal droppings were removed one at a time and immediately replaced by other trays already cleaned and relined with paper. Each soiled tray was put down over a large water-

tight pan of heavy galvanized sheet metal which also contained the waste can. Then the tray was cleaned by carefully folding up the newspaper lining and depositing it in the can with the aid of metal scraping tools kept in a bucket of disinfectant between operations. When the can was nearly full a quantity of disinfectant was poured over the contents, the cover was replaced, and the entire outer surface of the receptacle mopped with disinfectant. It was then carried to a steam cabinet where, with cover removed, it was subjected to steam under low pressure for two to three hours. From the steamer it was taken directly to the incinerator, emptied, and returned to the project room. Cleaning the small cages improvised from steam-table pans offered no difficulty. The chicks were transferred to clean pans and the soiled pans carried on a tray to an autoclave for sterilization.

In addition to preventing spread of infection to quarters outside the project room, it was essential to the success of the investigation that infections should not be transmitted from cage to cage within the room. The construction and placing of brooder equipment had been designed to prevent such spread by any activities of the chicks themselves or by manipulation of the clean-out trays. During such operations as treating, culturing, or examining chicks in successive cages, control was exercised chiefly by a thorough washing of the operator's hands with germicidal soap before moving from one cage to another. A system of terminal disinfection of cages was effective in preventing new groups of chicks from acquiring infections harbored by previous occupants of the brooders. The small cages were sterilized in the autoclave as usual; their hovers were sponged with chemical disinfectants. The commercial type brooders were dismantled; clean-out trays, wire mesh floors, food troughs, and side grilles were all taken to the steam cabinet. The few remaining parts which chicks could touch were sponged with disinfectant. To assist in the cleaning and disinfection of water troughs, which were difficult to handle when partially filled with contaminated water, a tank of adequate size, containing disinfectant and mounted on heavy swivel casters, was employed. Upon removal from brooders, water troughs were immersed in the disinfectant solution for at least 30 minutes before being rinsed and returned.

The feed used throughout was a commercial ration known as Chick Startena, Checkerettes, a product of the Ralston-Purina Mills. Feed and water were kept before the birds at all times and their quarters were always lighted, so that feeding was ad libitum day or night.

Cultures and bacteriologic techniques.—The strains of salmonella employed included fresh isolates from human and avian sources as well as a group of strains isolated originally from spray-dried egg powder (Solowey et al, 1947) and maintained in the lyophilized state for approximately 5 years.* The culture used most extensively was one of the latter group, hereafter designated *Salmonella typhimurium* 5609, which after two enteric passages through chicks was recovered from fecal cultures and then lyophilized in numerous vials in order that each experiment could be begun in standardized fashion with a vial of culture from the same drying.

On the day before administration to chicks a vial of dried culture was reconstituted with sterile distilled water. One capillary drop of the resuspended material was placed on an infusion agar plate to be streaked out as a check on purity of the culture; the remainder was discharged into 10 ml of warm beef heart infusion broth (Difco). After an incubation period of 18 to 24 hours at 37 C, serial decimal dilutions were prepared in cold sterile broth. Aliquots of appropriate dilutions were employed for infecting chicks and preparing plates for counting. Ordinarily both operations were completed within 30 minutes after the preparation of dilutions. On the basis of early experiments in which plate counting techniques were compared, as well as recommendations from the literature (Snyder, 1947; Crone, 1948), the method finally adopted was to calculate average counts from the results of cultures on 3 replicate plates each spread with 0.1 ml of a dilution yielding 30 to 300 colonies. When grouping data from several experiments, the practice followed here has usually been to round the numbers obtained from plate counts to whole powers of 10, although the plate counts themselves were recorded in two significant figures. It has been possible to do this without a large error because of the fortunate circumstances that viable counts of all overnight broth cultures of salmonella prepared by our standard method closely approximated 10^9 viables per ml. In the case of strains other than *S. typhimurium* 5609 any given figure for viable counts may be taken to represent a range no greater than that bounded by one-half the number and double the number stated.

Deliberate exposure of chicks to salmonella infection was accomplished by oral or intraperitoneal administration of cultured bacteria or by placing normal chicks in contact with infected birds. The method of oral inoculation adopted

* Kindly supplied by Dr. M. Solowey, Microbiology Research Div., Bureau of Agricultural and Industrial Chemistry, U. S. Dept. of Agriculture.

facilitated accuracy of measurement and avoided contamination of the operator's hands. A hypodermic needle and graduated tuberculin syringe were used to dispense 0.1 ml of culture dilution into the smaller end of a no. 5 gelatin capsule, which was then closed by application of the larger end of the capsule. The no. 5 capsules hold 0.1 ml of fluid but are dissolved rapidly, necessitating their individual filling and prompt administration. A brass tube, milled to hold snugly the large end of a capsule, was fitted with a solid brass plunger with which the capsule could be ejected.* It was a simple matter to place a loaded capsule in the tube, thrust it well down the throat of a chick, and discharge it by depressing the plunger.

Specimens of feces for the purpose of recovering salmonella from living chicks were obtained by inserting a small sterile cotton swab into the cloaca to a depth of not quite 2 cm and then withdrawing the swab. Blood for culture and other purposes was readily obtained by a heart puncture technique similar to that reported by Hofstad (1950). At least 1 ml of blood may usually be removed from a 4-day-old chick in this manner without causing death. Post-mortem specimens of intestinal contents and heart's blood were obtained for culture from all birds dying during experiments. All specimens were dated and labeled with the wingband numbers of the corresponding chicks; the results of cultures were recorded individually. Fecal samples were enriched overnight in freshly prepared tetrathionate broth (Difco), with brilliant green added to a concentration of 0.001%. Primary plating was to Difco brilliant green agar and/or SS agar. Extensive comparison of these two mediums in this laboratory (to be reported elsewhere) showed them to be equivalent for this type of work in respect to the number of positive cultures obtained. Suspected colonies on plates were fished to slants of Difco triple sugar iron agar (TSIA), and identifications were continued by standard methods, including examination of motility, biochemical behavior, and antigenic structure. For the latter purpose, slide agglutination tests were performed with bacterial suspensions usually derived from TSIA slants. The antigens were mixed with appropriate dilutions of serum containing antibodies to the anticipated 0 group, as well as serums directed against one or two heterologous 0 groups which served as control. Complete serologic typing was rarely considered necessary to establish the identity between an inoculated and a recovered organism. At least two colonies were fished from each plate at first examination, and if these did not yield the expected salmonella, the plates were refished at least once before being discarded as negative.

* This instrument was devised and fabricated by Dr. Foster Martin of the Dept. of Pharmacology, Tulane University, School of Medicine.

EXPERIMENTAL DATA

The studies described below were designed not only to furnish information regarding the characteristics of laboratory-induced infection of chicks with salmonella but also to provide a basis for other work dealing with attempted chemotherapy of such infections with various antibiotics. It seemed desirable initially to examine fairly extensively the behavior of one salmonella strain and subsequently to extend the observations to other strains as indicated. *S. typhimurium* strain 5609 was selected as a model because preliminary experiments showed it to be of moderate infectivity for young chicks and because it exhibited satisfactory in vitro susceptibility to the several antibiotics which it was planned to evaluate for therapeutic activity in these animals. This salmonella type is known to be a natural pathogen of rodents and other mammals including man, as well as of birds.

Estimation of number of bacteria in inoculums.—Plate counts of 37 overnight broth cultures of *S. typhimurium* 5609 prepared by our standard method over a period of 14 months gave an average of 94×10^7 viables per ml of culture. Although the period of incubation varied from 18 to 24 hours, the extreme values were 54×10^7 and 130×10^7. The range of 18 to 24 hours, it would appear, fell within the stationary phase of growth of this organism under the experimental conditions. This information made it possible to prepare suspensions of the organism of any given volume in which the viable count could be predicted with useful accuracy. Thus in 10 experiments where it was desired to administer to chicks doses

TABLE 1.—*Combined datd on oral infectivity of* S. typhimurium *5609 for one-day-old New Hampshire Red chicks (CL strain).*

Number of viables in inoculum	Number of chicks		Percent infected
	Examined	Infected	
10^6	15	15	100
10^4	15	15	100
10^3	15	15	100
10^4	39	38	97
10^3	59 (465)*	56 (449)*	95 (98.5)*
10^2	64	56	88
10	59	30	51
1-5	55	3	5

* The figures in parentheses show total experience with a dose of 10^3 bacilli of this strain. All birds were examined by cloacal swab culture 1 to 3 days after inoculation, and the demonstration of *S. typhimurium* in the feces was taken as evidence of infection.

on the order of 10^3 viables in 0.1 ml volumes, plate counts on aliquots of the test suspensions indicated that the actual doses fell in the range 1000 ± 300 for an average of 890.

Titrations of the oral infectivity of S. typhimurium *5609.*—The most extensive experience in determining the infectivity of salmonella strains has been obtained with the use of newly hatched chicks (24 to 48 hours out of the shell) which were inoculated prior to their first access to food and water. Such birds have been loosely referred to as "one-day-old" throughout these studies. The oral infectivity of *S. typhimurium* 5609 for one-day-old New Hampshire Red chicks (CL strain) has been studied in several separate titration experiments, the results of which are combined in table 1. In compiling the data for this table the figures respresenting the number of birds infected were taken from the results of cultures on the first cloacal swab specimens collected. In the more recent experiments this was a specimen taken 24 hours after inoculation, but in a number of experiments the first specimens were obtained 2 or 3 days after inoculation. The average infective dose under the conditions described was in the neighborhood of 10 viables, and a dose of 1000 viables produced demonstrable infection in at least 95% of a large number of chicks.

This degree of susceptibility to infection by the oral route was not restricted to a single breed and strain of young chicks, as is shown by two experiments summarized in table 2. In each experiment, newly hatched chicks of each of several strains, representing four of the heavy breeds, were obtained from the hatchery on the same day and given oral inoculations of *S. typhimurium* 5609 derived from the same overnight broth culture. The chicks of each strain were divided so that some chicks received 0.1 ml of 10^{-6} dilution, while others received 0.1 ml of 10^{-7} dilution. In one experiment the larger inoculum contained 95 viables; in the second experiment, 54 viables. Cloacal swab specimens were obtained for culture either

TABLE 2.—*Oral infectivity of* S. typhimurium *5609 for one-day-old chicks of various breeds and strains.*

Breed	Strain	Number of viables in inoculum	Number positive /number examined*	
			Days after inoculation	
			1	2
Barred Plymouth Rock	L	95		8/10*
		9		5/10
		95		8/10
		9		9/10
		54	16/20	
		5	13/21	
New Hampshire Red	CL	95		7/10
		9		3/10
	K	54	21/21	
		5	16/22	
	S	95		4/10
		9		2/10
Rhode Island Red	B	54	18/20	
		5	5/21	
	S	95		10/10
		9		10/10
White Plymouth Rock	B	54	20/20	
		5	4/21	
	S	95		7/10
		9		4/10
Totals		54	75/81	
		5	38/85	
		95		44/60
		9		33/60
Grand totals		50–100	119/141 (84.4%)	
		5– 10	71/145 (49%)	

* In this and the following tables, numerator = number of chicks yielding positive fecal cultures for the salmonella inoculated; denominator = number of chicks examined culturally.

TABLE 3.—*Oral infectivity of* S. typhimurium *5609 for New Hampshire Red (CL strain) chicks of various ages.*

Age of chicks at time of inoculation	Days after inoculation until culture	Number positive/number examined				
		Number of viables in inoculum				
		10^8	10^4	10^4	10^2	10
4 days	2	7/10	7/10	9/10	1/10	0/10
	6	10/10	9/10	9/10	4/10	0/10
7 days	2	8/10	1/10	1/10	0/10	0/10
	6	8/10	1/9	2/10	0/10	0/10
2 weeks	2	2/10	1/10	0/10	0/10	0/10
	6	3/10	1/10	0/10	0/10	0/10
3 weeks	2	8/10	1/10	0/10		
	6	9/10	4/10	0/10		
4 weeks	2	1/11	2/11			
	6	2/11	2/11			
7 weeks	2	2/9		0/9		
	6	3/9		0/9		

one or two days following inoculation. With one possible exception (strain NHR-S) the results were about what might be expected on the assumption that no striking strain differences existed as regards susceptibility to infection by the oral route.

The influence of age on susceptibility is demonstrated in table 3. A large group of (NHR-CL) chicks of a single hatch was set aside in a special brooder and drawn upon at intervals over a 7-week period for repeated titrations of *S. typhimurium* 5609. Ten chicks were given oral doses of each dilution tested, and cloacal swab specimens were collected at 2 and 6 days after inoculation. Somewhat decreased susceptibility of the birds was apparent even at 4 days of age as compared with the expected results (see table 1) in one-day-old chicks of this strain; thereafter only large doses produced detectable infection, and that, irregularly.

The oral infectivity of other types of salmonella for young chicks.—As far as has been determined the behavior of *S. typhimurium* 5609 in chicks is typical of many salmonella types rather than exceptional. The oral infectivity of 25 additional strains, with some duplication of types, has been titrated in one-day-old chicks by our standard method. One strain of *Salmonella senftenberg* isolated from egg powder and one strain of *Salmonella choleraesuis* var. *kunzendorff* from a human source appeared to be noninfectious to chicks. The results obtained with the remaining 23 cultures are summarized in table 4, where it may be seen that the average infective dose for the majority of strains tested was on the order of 10 viable bacilli, as in the case of *S. typhimurium* 5609. In this series no marked differences in the results of the titrations were noted whether the first cloacal swab cultures were taken after an interval of 1 day (3 strains), 2 days (19 strains) or 3 days (1 strain) following oral inoculation.

Enteric infection following intraperitoneal inoculation.—Although the major portion of this study was devoted to infections introduced via the oral route, for the sake of comparison 3 experiments were performed in which *S. typhimurium* 5609 was inoculated intra-abdominally in one-day-old chicks of the standard strain, using groups of 6 to 7 chicks for each culture dilution. Cloacal swab specimens were collected on the day after inoculation and from survivors at intervals thereafter. Table 5 summarizes the results of cultures taken 24 hours after inoculation. It is clear that the salmonella began to be excreted in the feces shortly after intraperitoneal inoculation and that measured by the incidence of positive cloacal swabs the culture was probably more highly infective by this route than when administered orally. Most of these chicks died within a few days in contrast to the relatively low death rate following oral inoculation (vide infra).

Bacteremia following oral inoculation of chicks.—Cultures of heart's blood taken post-mortem from inoculated

TABLE 4.—*Oral infectivity titrations in one-day-old chicks of 23 strains of salmonella representing 10 types from 4 serologic groups.*

Strain of salmonella tested	Source	Kauffman-White group	Number positive/number examined						
			Number of viables in inoculum						
			10^6	10^4	10^3	10^2	10^*	1	<1
S. paratyphi B 6714	Egg	B		6/6	6/6	6/6	6/6	1/6	1/8
S. paratyphi B 6-57	Egg	B		6/6	6/6	3/6	6/6	1/6	0/8
S. paratyphi B 944	Egg	B		6/6	6/6	6/6	3/6	0/6	0/7
S. paratyphi B 275 E	Human	B		4/6	4/6	6/6	2/6	3/6	0/7
S. typhimurium A-6087	Egg	B	5/5	5/5		4/5	3/5	1/5	
S. typhimurium (Smith)	Human	B		6/6	6/6	2/6	0/6	1/6	0/6
S. montevideo 4819	Egg	C_1	5/5	5/5		5/5	1/5	0/5	
S. montevideo B-33	Human	C_1	6/6	6/6		6/6	6/6	0/6	
S. tennessee 5977	Egg	C^1	6/6	6/6		5/6	1/6	0/6	
S. tennessee 5307	Egg	C_1	6/6	6/6		6/6	6/6	1/6	
S. tennessee 5499	Egg	C_1	6/6	6/6		6/6	6/6	0/6	
S. panama CM 1118	Egg	D		6/6	5/6	6/6	1/6	1/6	0/6
S. enteritidis	Stock culture	D		6/6	5/6	5/6	2/6	1/6	0/6
S. anatum 5171	Egg	E_1	6/6	6/6		6/6	4/6	2/6	
S. anatum 6478	Egg	E_1	6/6	6/6		6/6	3/6	0/6	
S. anatum 5343	Egg	E_1		6/6	5/6	6/6	0/6	0/6	0/6
S. meleagridis 5288	Egg	E_1	6/6	6/6		6/6	6/6	0/6	
S. meleagridis 5389	Egg	E^1	6/6	6/6		6/6	4/6	0/6	
S. meleagridis M-101	Human	E_1	6/6	6/6		6/6	4/6	0/6	
S. meleagridis 5644	Egg	E_1		6/6	6/6	6/6	4/6	1/6	
S. meleagridis 5485	Egg	E^1		6/6	6/6	4/6	0/6	0/6	0/6
S. give K-93	Human	E_1		6/6	6/6	6/6	4/6	0/6	0/7
S. senftenberg 3252 S	Egg	E_2	6/6	6/6		6/6	6/6	6/6	
Totals			70/70	134/136	61/66	124/136	78/136	19/136	1/67
Percent infected			100	98.5	92.4	91.2	57.3	14.0	1.5

* 10 = 5 to 20.

birds indicated that more than a purely enteric infection was involved, at least in fatal cases. In table 6 are combined the data from three experiments in which blood for culture was obtained ante-mortem at intervals following oral inoculation with doses of varying size.

TABLE 5.—*Enteric infection with* S. typhimurium *5609 in one-day-old chicks (NHR-CL strain) following intraperitoneal injection.*

Number of viables in inoculum	Number of chicks		Percent infected
	Examined	Infested	
10^6	12	12	100
10^2	18	17	94
10^2	18	18	100
10	19	15	79
1-5	19	9	47

These may indicate the usual course of events with regard to invasion of the blood stream. All chicks were bled from the heart by the same technique, but all results are not strictly comparable because of differences in the amounts of blood per sample and the culture methods employed. In experiment 3, for example, only 0.1 ml of blood was taken per bleeding and this was spread on the surface of an SS plate; whereas the bleedings of 0.1 to 0.5 ml obtained from birds in experiments 1 and 2 were cultured in broth. Nevertheless, it seems clear that following oral administration of one or more infective doses

TABLE 6.—*The presence of* S. typhimurium *5609 in blood and feces of chicks after oral inoculation.*

Exp. no.	Number of viables in inoculum	Specimen cultured	Number positive/number examined									
			Days after inoculation									
			1	2	3	4	5	6	7	14	21	28
1	10	Blood	0/10	3/10	6/9	9/9	4/5	3/3				
		Feces	10/10	10/10	9/9	9/9	5/5	3/3				
2	10^2	Blood				13/13			8/11	3/8	0/7	0/7
		Feces	13/15			15/15			10/11	8/8	6/7	2/7
3	10^4	Blood	0/11	3/10	2/8	4/8	4/8	3/7	1/6	0/6	0/6	1/6
		Feces	11/11	10/10	8/8	8/8	8/8	7/7	6/6	6/6	6/6	5/6
Totals		Blood	0/21	6/20	8/17	26/32	8/13	6/10	9/17	3/14	0/13	1/13
		Feces	34/36	20/20	17/17	32/32	13/13	10/10	16/17	14/14	12/13	7/13

of this particular strain of salmonella a bacteremia develops which, although not detectable 24 hours after inoculation, is readily demonstrated in the majority of chicks by the fourth day. Thereafter recoveries take place so that by the end of the third week the blood of most surviving chicks no longer yields positive cultures. The infecting organisms may be found in the feces earlier, more regularly, and over a longer period than in the blood. This same relationship seems to hold true with other types of salmonella which have been tested.

The duration of enteric infection following oral inoculation.—The persistence of infection in chicks, as indicated by positive cultures of feces, has not been accurately determined because of our limited housing facilities, but some observations bearing on this point were made. Only 1 of 40 chicks infected with a strain of *Salmonella montevideo* (B-33) was found to be eliminating salmonella in the feces after 50 days, and about half of the birds had become negative by the end of the third week. The average duration of enteric infections with with *S. typhimurium* 5609 is certainly greater than that of *S. montevideo* B-33 but adequate sampling has not been made beyond the 4th week. In experiments involving the oral administration of approximately 1000 viables, *S. typhimurium* 5609 was recovered from cloacal swabs of over 95% of 787 birds inoculated up to 2 weeks previously, and from at least 85% of 121 chicks examined through the 4th week after inoculation.

Evidence that early positive cloacal swab cultures signify infection.—Infection is ordinarily defined as the state produced by the entrance into the body and multiplication therein, of pathogenic microorganisms. As a matter of fact, it is rarely considered necessary to prove multiplication before describing a process as infectious, but since we were dealing with a relatively unfamiliar type of experimental infection we thought it worth-while to make the demonstration. Eventual multiplication of the orally inoculated salmonella in chicks is shown clearly by the data in table 6. Birds given oral doses of 10 viables yielded repeatedly positive cultures of both blood and feces. Since the material cultured daily was only a small sample from the total blood volume and intestinal contents, there can be little doubt but that substantial multiplication occurred.

The real point at issue was whether positive cultures from cloacal swabs obtained at 24 hours after inoculation indicated infection or merely the recovery of a portion of the inoculum which was still in passage through the alimentary tract. This could be answered in part by records of several groups of chicks which were inoculated

with small numbers of salmonella and cultured frequently thereafter. The regularity with which chicks found positive after one day remained so for a period of days or weeks is incompatible with the idea of a surviving, but nonmultiplying, inoculum. By contrast, chicks which were insusceptible by reason of age were never positive at any time. Nevertheless, experiments were performed to compare the numbers of bacteria inoculated and excreted. To obtain an approximation of the numbers of salmonella in the feces, cloacal swab specimens were twirled into measured volumes of saline to free most of the fecal matter. Decimal serial dilutions in saline were made from the original suspension, and portions of each dilution were inoculated into tetrathionate broth, which was then processed as usual for the recovery of salmonella. By assuming that positive cultures could result from even one viable salmonella cell reaching the tetrathionate broth and by making the appropriate calculations, the minimum probable number of these organisms in the fecal specimen could be approximated (cf. Gibbons and Moore, 1946). From swab specimens taken 24 hours after oral inoculation of chicks the estimated numbers of salmonella recovered frequently exceeded those of the inoculums, in some instances by several hundredfold. It was clear that proliferation of the organisms was well under way within the first day of their residence in the avian host.

Morbidity and fatality from experimental salmonella infections.—In view of the severity of many of the reported outbreaks of paratyphoid infection in fowls (e.g., Edwards et al, 1948), it was somewhat surprising to find in this study that physical signs of infection were usually mild following experimental exposure by gavage or by contact with inoculated chicks. Despite bacteriologic evidence of continued infection and a moderate number of deaths, the majority of the chicks were alert, ate well, and appeared to be making normal growth. The "white diarrhea" associated with pullorum disease was notably absent; nevertheless, after long observation it was concluded that most of these chicks exhibited slight diarrhea. This could easily escape detection because of the apparent good health of the birds and because the excreta did not tend to accumulate about the vent or cling to the ventral surface of the chick in most instances. However, it was noted that within one to three days after administration of an infecting dose there occurred in most of the inoculated chicks a period of frequent, forceful emissions of a clear, watery fluid from the vent. Beyond this mild type of diarrhea, observation of the infected chicks revealed little except for occasional prostration and death. Temperatures were not taken.

Since the majority of chicks were held for a relatively short time only, with little uniformity in the periods of observation, and since small losses, particularly during the first week of life, are normal in the brooding of hatchery chicks, the specific fatality from experimental salmonella infection could be usefully described only by comparing a well-definined group of infected chicks with a similar group of uninoculated controls. There were available for this purpose records of 92 chicks from control groups and 112 chicks which had been administered gavage doses of 1000 viable *S. typhimurium* 5609, in each of which categories survivors had been observed at least 8 days (for mean observation periods of 12 and 14 days after inoculation, respectively). In the control group there were 8 deaths, a gross mortality of 8.7%. In the infected

TABLE 7.—*Chronologic distribution of deaths among uninfected control chicks and chicks orally inoculated with 1000* S. typhimurium *5609.**

Days after inoculation	Number of deaths among		
	Infected chicks		Control chicks
	Total	Specific†	Total
1	0	0	0
2	1	1	2
3	2	1	2
4	2	2	0
5	1	1	0
6	7	6	1
7	7	7	2‡
8	4	4	1
9	5	4	0
10	1	1	0
13	1	1	0
15	1	0	0
48	1	0	
Totals	33	28	8
Number examined	112	112	92
Percent dying	29	25	8.7

* Only groups of chicks in which survivors were observed for at least 8 days were counted. Mean observation times were: infected group, 14 days; control group, 12 days.
† *S. typhimurium* (and no other salmonella) isolated from both heart's blood and intestinal contents.
‡ *S. pullorum* was isolated from the heart's blood of both these chicks.

group there were 33 deaths (29%); *S. typhimurium* (and no other salmonella) was isolated from both intestinal contents and heart's blood of 28 of the dead chicks. Of the other 5 which died, 1 was inadvertently not cultured, 2 were negative for salmonella, 1 yielded *S. pullorum* as well as *S. typhimurium*. From 1 the expected organism was recovered from the intestinal contents but not from the heart's blood. The 28 fatalities with doubly positive bacteriologic findings are referred to in table 7 as "specific" deaths (in contrast to the 33 "total" deaths) and from them a case fatality rate of 25% may be calculated. However, it is still possible that some of these 28 deaths might have occurred in the absence of any salmonella infection. It will be noted further that all deaths in the control group took place in less than 12 days. Twenty-seven of the "specific" deaths among infected chicks also occurred within 12 days, a fatality rate of 24.1%, which it is believed may be fairly compared with the death rate in the control group (8.7%). The difference between the two rates (15.4%) is approximately three times the standard error of the difference (=5.3%, $P=0.0036$) and is regarded as the best available measure of specific fatality from experimental oral infection with *S. typimurium* 5609. Insufficient data are on hand at present to warrant comparison of the relative virulence of different strains of salmonella in the same manner that the relative infectivity has been examined, but it may be stated that for the majority of strains studied so far the incidence of specific fatalities is low during the first week following oral inoculation.

Our limited data show that fatality rates are much higher following intraperitoneal inoculation of one-day-old chicks with *S. typhimurium* 5609. Table 8 was compiled from 2 titration experiments in which survivors were observed for 3 days in one case and 10 days in the other. Despite the short periods of observation and small numbers of animals involved it is apparent that the test organism had a different order of virulence when injected intra-abdominally from that observed following gavage inoculation.

The development of antibodies following oral inoculation.—No extended effort

TABLE 8.—*Chronologic distribution of deaths after intraperitoneal inoculation of one-day-old chicks with varying doses of* S. typhimurium *5609.*

Days after inoculation	Number of specific deaths*						
	Number of viables in inoculum						Totals
	10^3	10^4	10^5	10^6	10	1	
1	4	2					6
2	1	6	11	3	3	1	25
3		1		3	1	2	7
4				3			3
5				1			1
6		1			1		2
Total deaths	5	10	11	10	5	3	44
Number inoculated	5	12	12	12	12	12	65
Percent fatality	100	83	92	83	42	25	68

* *S. typhimurium* was recovered in each instance from heart's blood and intestinal contents. Two additional chicks died with negative bacteriologic findings.

has been made to secure data concerning this point, but a few observations made incidental to other work seem worthy of mention. In one experiment, two-day-old chicks were each given an oral dose of 8600 viable *S. typhimurium* 5609. At intervals thereafter cultures of blood and feces were taken. The birds were bled 35 days after inoculation and their serums were tested for antibodies. In the serums from 4 of the 5 birds anti-

TABLE 9.—*Cultural and antibody data on chicks infected orally with 8600 viable* S. typhimurium *5609.*

Chick number	Days after inoculation					
	1–7		28		35	
	Fecal culture	Blood culture	Fecal culture	Blood culture	Fecal culture	Anti-H titer*
2312	+	+	0	0	+	160
2315	+	0	+	0	0	0
2316	+	+	+	0	+	40
2317	+	0	+	0	+	160
C-1	+	+	+	+	+	320

* Anti-H titers are expressed as reciprocal of the final dilution of serum giving agglutination in macroscopic tube tests employing formalinized broth culture as antigen.

bodies against the H antigenic complex were detectable, with the anticipated variation in titers (table 9). Additional scattered demonstrations of the appearance of homologous antibodies following experimental infection have been made.

DISCUSSION

On examining the extensive literature on experimental avian salmonellosis, we have found surprisingly little quantitative bacteriologic data of the type presented here. In the study of *S. pullorum* or *S. gallinarum* infections which has occupied many workers, despite the frequent practice of administering orally large numbers of organisms (often derived from a mixture of strains), fecal cultures were usually not recorded. Some investigators, however, have laid stress on the importance of bacteriologic examination of the intestinal contents in attempting to determine the presence of infection, particularly if salmonella types other than *S. pullorum* are involved (cf. Posell, 1942; Hinshaw and McNeil, 1943; Bushnell and Porter, 1945; Gibbons and Moore, 1946; Gauger and Greaves, 1946; Chase, 1947). There is general agreement that for this purpose preliminary enrichment in a fluid medium such as tetrathionate broth is indispensable. For subculture, in order to obtain isolated colonies for further study, various plating mediums have been used with success, including the SS agar and brilliant green agar employed in this study (e.g., Mallmann et al, 1942; Hinshaw and McNeil, 1943; Bushnell and Porter, 1945; Gauger and Greaves, 1947). We have found, by trial with measured quantities of salmonella added to fecal suspensions, that numbers of organisms as few as even single viables can be detected by the enrichment plus plating method providing that the salmonella are not overgrown by other components of the inoculum, e.g., *Proteus*, which can proliferate in the tetrathionate broth. The procedure used in the present investigation is therefore a very sensitive method for the detection of enteric salmonella infection.

With the aid of techniques comparable to those just discussed, several salmonella types have been recovered from the intestinal contents of orally inoculated young or mature turkeys (Hinshaw and McNeil, 1943; Pomeroy and Fenstermacher, 1943; Gauger and Greaves, 1946 and 1947) and chickens (Gibbons and Moore, 1946; Buxton and Gordon, 1947). The data obtained in the present study have extended the existing knowledge concerning the pathogenesis of salmonella infections in very young chicks and have made it possible to analyze some of the basic features. It has been clearly demonstrated (for salmonella types from sever-

al of the Kauffman-White groups) that these animals are susceptible to controlled oral infection by much smaller numbers of organisms than heretofore employed, with the consequent development of a syndrome which in several important respects resembles natural salmonella infection of man. Thus although some of the birds develop a fatal form of the infection, others (the majority) resist the disease and eventually return to health. A few apparently become carriers for relatively long periods. The oral inoculation of very young chicks provides the best means with which we are acquainted for maintaining in the laboratory large numbers of demonstrably salmonella-infected animals. The ease of obtaining daily (or more frequent) cultural evidence of infection or lack of it renders such birds particularly suitable for studying bacteriologically the influence of chemotherapeutic agents on the course of salmonella infection.

Although inoculation of very young chicks by either the oral or intraperitoneal route produces bacteremia in the majority of animals and also leads to an enteric phase with elimination of salmonella in the feces, the infections produced differ in that among birds inoculated intraperitoneally the death rate is higher for any given dose and the time until death is shorter. Severens et al (1944), while studying resistance to pullorum disease used chicks as young as one day old, gave relatively large quantities of bacteria by both oral and subcutaneous routes, and determined the size of dose by enumeration in a counting chamber. They performed blood culture studies with results which are consistent with our findings; no fecal cultures are recorded. Others have used the intra-abdominal route for injecting measured numbers of salmonella but have not reported the results of cultures made, if any (e.g., Little, 1948; Pomeroy et al, 1948; Bell, 1949).

It is altogether probable that strain differences among chickens in susceptibility to salmonella other than *S. pullorum* do occur (cf. Lambert, 1932; Hutt and Scholes, 1941; Scholes and Hutt, 1942; Severens et al, 1944; Bell, 1949), but the results of our experiments with random selection of stock—even though the numbers are not large—indicate that obtaining suitably susceptible birds should offer little difficulty to workers who wish to conduct similar experiments with infections induced by oral administration of small doses of salmonella. It is necessary, however, to use very young chicks because of the demonstrated rapid acquisition of resistance to oral infection with age (see also Holtman and Fisher, 1947). The development of natural resistance as the chick grows older has been a deterrent to certain types of immunologic study which we considered to be of interest. For example, it was impossible to measure the resistance of chicks recovered from an initial enteric infection to subsequent challenge with salmonella of the same or different types administered orally since normal chicks of the same age were likewise resistant to the challenge doses.

In chemotherapeutic and other experments to be reported later, as well as in these infectivity studies, we have chosen to quantitate bacterial suspensions by estimating the number of viable units rather than by the use of microscopic enumeration. Since in the preparation of suspensions by the simple expedient of growing broth cultures overnight there were variations in the incubation period amounting to a substantial multiple of the generation time with never more than a twofold variation from the mean viable count, it is hardly to be doubted that the proportion of viable

to nonviable bacteria varied considerably from experiment to experiment. We have no evidence that such variation had any effect on the infectivity or pathogenicity of inoculums. According to other investigators of counting techniques the choice of pour-plate, surface colony, agar strip, or roll-tube method of performing viable counts is not as important as accuracy and constancy of technique combined with sufficient replication of plates or tubes (Snyder, 1947; Crone, 1948; Stirling et al, 1950). In preliminary work in this laboratory pour-plate and surface colony plate counts of a considerable number of salmonella suspensions were compared. The agreement was satisfactory, but numerically higher values were obtained by the surface colony method in a significant proportion of trials. Since this method was considered to have the practical advantages of greater speed and simplicity in performance, both in setting up and in counting, it was chosen as the standard procedure.

The validity of the data obtained in the infectivity titrations may be questioned because of the possibility of spread of infection by contact within the cages. Ideally, of course, the birds should have been housed individually rather than by groups, but this was wholly impractical if the methods were to find any wider application. As has been mentioned previously, all birds of a group receiving the same dose of a particular culture were housed together either in a brooder or an enamelware pan so that opportunities for contact infection were present. It might therefore be postulated that one particularly susceptible bird could become infected from a dose which would be noninfectious to its cage mates and, after incubation and multiplication of the organisms in its body, might transmit the infection to other chicks by shedding large numbers of salmonella in its feces. From the following observations it appears, however, that such a sequence of events could not be fairly invoked to explain the results when nearly all the birds in a given group were shown to yield positive cloacal cultures within 24 hours after oral administration of very small numbers of salmonella (see tables 2 and 6).

Six one-day-old chicks were given by mouth approximately 40 viables of *S. typhimurium* 5609; six birds received a similar dose of *S. montevideo* B-33. Each inoculated bird was placed in a separate enamelware pan together with 5 uninoculated chicks. Four of the 6 birds inoculated with either strain yielded the salmonella from cloacal cultures taken the next day; one additional inoculated chick yielded its first positive fecal culture only after 48 hours of infection. Thus a total of 9 out of 12 birds receiving 40 viable salmonella could each be shown to be infected, even when they were not in contact with any other inoculated chicks. The incidence of infection under these conditions agrees well with the findings on cloacal cultures taken after 24 hours from birds given similar inoculums but housed together in groups. Furthermore, among 40 uninoculated birds exposed to the 8 chicks whose infection was manifest within 24 hours of inoculation, only two could be shown to have acquired infection during the first day of contact. By 48 hours, however, a total of 24 exposed birds were shown to have developed contact infections, and during a week of exposure nearly all of the 40 birds became infected. Because of these and other data it has been thought unwise to calculate 50% end points or to otherwise regard the infectivity studies as exactly quantitative. Nevertheless, it is believed that the titration method employed does give useful information re-

garding the oral infectivity of salmonella strains for birds.

SUMMARY AND CONCLUSIONS

Chicks were inoculated orally or intraperitoneally with varying numbers of viable salmonella. Evidence of infection was obtained by bacteriologic examination of specimens obtained by cloacal swab or cardiac puncture.

It was found that one-day-old birds could be readily infected by the oral route with very small doses of organisms belonging to any of several of the Kauffman-White serologic groups. For the purpose of analyzing certain features of the infectious process, additional experiments were performed which involved chiefly a single strain of *Salmonella typhimurium*. Susceptibility to infection by the oral route decreased rapidly with advancing age of the birds. Animals from any of several breeds could be used satisfactorily providing they were of the proper age. The case fatality rates were not high although the chicks frequently showed bacteremia. The presence of salmonella in the feces was demonstrated earlier, more regularly, and over a longer period of time than in the circulating blood. The duration of enteric excretion appeared to vary depending on the infecting strain and the individual bird affected, with some infections persisting for at least several weeks. Following oral infection antibodies appeared in the serum.

A similar type of infection could be induced by intraperitoneal injection of salmonella, although a higher fatality rate was observed to follow.

The ease with which young chicks can be infected by the oral route with small numbers of organisms of many salmonella types and the regularity with which the organisms can be demonstrated in fecal cultures taken over a period of many days, despite the benign course of the disease in most animals, indicate that this experimental method should be applicable to the study of additional aspects of salmonellosis.

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A SEROLOGICAL STUDY OF CERTAIN MEMBERS OF THE AEROBIC NONPATHOGENIC NEISSERIA GROUP

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The American scheme of classification —as indicated in *Bergey's Manual of Determinative Bacteriology* (Breed et al, 1948)—recognizes 6 distinct species of aerobic, nonpathogenic neisseria. The similar morphology and uniform nonpathogenicity prevailing among these organisms, coupled with conflicting data on description of pigment, colonial structure, and biochemical activity, have raised doubts as to the validity for recognition of this number of separate species. In contrast to this scheme of species differentiation, some British workers (Wilson and Miles, 1946) support the establishment of a single species, *Neisseria pharyngis*, for all of the saprophytic neisseria, with the possible exception of *Neisseria catarrhalis*. These divergent views on the systematic relationship of the saprophytic neisseria emphasize the need for their further characterization.

The effect of basal mediums on carbohydrate utilization was recently investigated for a possible explanation of inconsistencies noted in the fermentative capacity of saprophytic neisseria (Hajek et al, 1950). These investigators observed significant differences in the fermentation of carbohydrates incorporated in different basal mediums and advocated the establishment of one basal medium for such biochemical studies of neisseria. There is general agreement concerning the failure of *N. catarrhalis* to produce acid in any of the carbohydrates usually employed for biochemical differentiation. The fermentation of mannite supposedly characteristic of *Neisseria perflava* (Breed et al, 1948) was not confirmed by Pelczar and Nemes (1949) for their strains of pigmented neisseria which fermented glucose, fructose, maltose, and sucrose. The systematic position of *Neisseria sicca* has been questioned, and it has been suggested that the sicca species is merely a rough variant of one of the nasopharyngeal cocci fermenting glucose, maltose, and sucrose (Wilson and Miles, 1946).

There has been little investigation of the serological relationship of members of the aerobic, nonpathogenic, gram-negative cocci. Wilson and Miles (1946) attributed, as the primary reasons, autoagglutination and lack of stable homogeneous suspensions in saline. In the classical presentation of Elser and Huntoon (1909), these authors reported (1) that none of their chromogenic species showed any serological relationship to the meningococcus, gonococcus, or *N. catarrhalis* by agglutination tests, (2) that their three chromogenic groups were distinguishable from each other by agglutination tests, and (3) that strains of *N. sicca*, *N. catarrhalis*, and occasionally members of the chromogenic groups autoagglutinated or showed a tendency to spontaneous sedimentation in saline and normal serum. Branham (1930) reported *Neisseria flavescens*, which is chromogenic and nonfermentative, to

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be a serologically homogeneous group.

The study reported here is a serological investigation of some nonpathogenic neisseria to establish any correlation between this and other characteristics.

MATERIALS AND METHODS

Cultures.—Of the 35 neisseria strains reported herein, five strains (9746, 9913, 8193, 8176, and 7900) were obtained from the American Type Culture Collection; two strains (2AMS and 1485) from the Army Medical Center; six strains (31R, 14, 22, 28, 8, and 17) from the Johns Hopkins University; two strains (155 and 157) from Dr. S. E. Branham, National Institutes of Health; two strains (MF1 and MF2) from Dr. M. Frobisher, Jr., Communicable Disease Center, U.S.P.H., Atlanta; five strains (1196, 0601, 0765, 0650, and 01197) from McGill University; and one strain (54) from State University of Iowa. Three strains (GP3, GP16 and F) were isolated from the bacterial flora of the pharyngeal region of normal guinea pigs, and the remainder (7925, 12, 24, 10-1, 20-1, 55, 4, 3-1, and 4-2) were isolated from the throat of normal humans at the University of Maryland. Cultures were given species names according to their fermentation reactions and pigmentation. Some instances of deviations from typical reactions (Breed et al, 1948) were encountered; these cultures were assigned a species name most nearly associated with the atypical reactions.

Stock cultures were maintained on cystine trypticase agar at 30 C and were transferred at three-week intervals. Before a culture was employed in a test, it was transferred daily on trypticase soy agar for at least one week and was maintained thereafter on this medium during the test period.

Cultures of the collection were examined and found to conform to the morphological and cultural description of neisseria. Colonies of each organism grown on trypticase soy agar were oxidase-positive after 48 hours' incubation at 35 C (para-amino dimethylaniline monohydrochloride reagent). On each culture a microscopic slide agglutination test (Branham, 1945) was performed with polyvalent antimeningococcic horse serum. Controls of normal horse serum and saline were included with each test. There was no evidence of any serological relationship to the meningococci.

Carbohydrate fermentation and pigmentation.—Strains of the collection were tested for their fermentative capacity on five carbohydrates, namely, glucose, fructose, maltose, sucrose, and mannite. Each carbohydrate was made up as a 10% solution in distilled water, sterilized by Seitz filtration, and then added to previously sterilized cystine trypticase agar to give a final concentration of 0.5%. Results were recorded after 48 hours and again after 7 days' incubation at 35 C. To determine pigment production, the growth on Loeffler's slants was observed after an incubation of 48 hours and 7 days at 35 C.

Serological methods.—Eighteen cultures were selected from the collection on the basis of fermentation reactions and pigmentation. An antiserum was prepared for each culture by intravenous injection of rabbits with living suspensions. The antigens were saline suspensions of growth washed from a 20- to 24-hour trypticase soy agar slant with 5 ml of a 0.85% buffered sodium chloride solution (pH 7.4). The cell suspension, which was prepared on the day of injection, was transferred aseptically to a sterile serum bottle, shaken on a Kahn shaker for 10 minutes, and then diluted with buffered saline to the desired density using a McFarland nephelometer standard for comparison. Rabbits (in duplicate) were injected with increasing doses of antigen over a two-week period. During the first week 0.5 ml of a 900 million cell suspension was given on the first day and 1 ml on the second and third day. Rabbits were rested for the remainder of the week. The second week's inoculum consisted of 0.5 ml of a 1200 million cell suspension the first day, 1 ml the second day, 1.5 ml the third day and 2 ml the fourth day. A trial bleeding was made on the fifth day following the last injection, and rabbits with satisfactory titers were exsanguinated by cardiac puncture. Occasionally, a higher titer antiserum could be produced by injections for an additional week.

The tube agglutination test recommended for the meningococci was followed except for a few minor modifications (Branham, 1945). Antigens were prepared as indicated above and diluted with buffered saline to a density corresponding to no. 3 McFarland nephelometer standard (900 million cells per ml). The agglutination test employed the usual procedure of doubled dilutions of antiserums (1:20 to 1:10,240) and also the intermediate dilutions of 1:200, 1:400, 1:800, 1:1000, 1:1600, and 1:2000. Doubled dilutions (1:20 to 1:640) of normal rabbit serum and a saline control were included with each test. Results were recorded without shaking the tubes. The titer was ascertained by the degree of clearing in the supernatant liquid and the amount of agglutinated cells.

The agglutinin absorption technique was employed to study the serological relationship of certain strains observed to cross-agglutinate. The absorption method used was essentially that de-

TABLE 1.—*Biochemical and pigmentation studies on strains of aerobic nonpathogenic neisseria.*

Species designation	Culture number	Fermentations					Pigmentation
		Glucose	Fructose	Maltose	Sucrose	Mannite	
N. flavescens	157, 155, 9746	−	−	−	−	−	Yellow
N. perflava	2AMS, 54, 12, 24, 31R, 10-1,22, 20-1, 0765, 0601	+	+	+	+	−	Yellow to caramel
N. perflava	14, 17, 28, 8	−	+	+	+	−	Yellow to caramel
N. perflava	1196	+	+	+	V	−	Yellow
N. perflava	7925	+	+	−	+	−	Yellow
N. flava	55, 3-1, 4	+	+	+	−	−	Yellow
N. sicca	1485, 4-2	+	+	+	+	−	White
N. sicca	9913	V	−	V	V	−	White
N. catarrhalis	8176, 8193, 7900, 0650, 01197, MF1, MF2	−	−	−	−	−	White
N. species	GP3, GP16, F	−	−	−	−	−	Yellow

+, acid; −, no change or alkaline; V, variable.

scribed by Stuart et al (1940) in the serological identification of coliform bacteria.

RESULTS

Biochemical and pigmentation studies.—The results of biochemical and pigmentation studies are given in table 1.

All the strains of *N. catarrhalis* were comparable in their morphological and biochemical characteristics, but were sufficiently different from other strains of saprophytic neisseria in their lack of fermentative capacity and absence of pigmentation to constitute a distinct group. The flavescens strains and guinea pig isolates (GP3, GP16, and F) demonstrated a lack of biochemical activity which differentiated them from other chromogenic species. The chromogenic, fermentative strains varied in their ability to utilize carbohydrates and showed slight differences in pigmentation. The fermentation of sucrose was considered in this study as a biochemical differentiation between the perflava and flava species. The flava strains were negative in sucrose. The two fermentative strains 1485 and 4-2 were nonchromogenic and gave typical carbohydrate reactions for *N. sicca* (Breed et al, 1948).

Serological studies.—The tube agglutination results of 35 aerobic nonpathogenic neisseria in 16 prepared antiserums are given in table 2. An agglutination titer of less than 1:200 was arbitrarily considered as not significant.

As may be seen from table 2, strains of *N. flavescens* (157, 155, and 9746) formed a distinct serological group. No cross agglutination between this group and any of the other strains of neisseria tested was encountered, except for a weak reaction with *N. perflava* 7925 by the antiserum prepared against strain 155. Strain 155 was agglutinated in a dilution of 1:200 by the antiserum prepared against *N. perflava* 14; however, strain 9746, which was identical in all other respects to strain 155, demonstrated no cross agglutination with antiserum 14. These strains were shown to possess similar antigen structure by agglutinin absorption experiments.

The guinea pig isolates (GP16, GP3, and F) were comparable in morphological and biochemical characteristics to the flavescens strains but gave no evidence of serological relationship (table 2). Except for the agglutination of strain GP16 in the antiserum 7925 to a titer of 1:320, the guinea pig strains showed no serological relationship to any of the human strains employed in this study. Rabbits inoculated with GP16 failed to produce a titer of more than 1:200. Furthermore, when other strains of the collection were crossed with antiserum GP16, no antigenic relationship was demonstrated.

Perflava strains, which have been

TABLE 2.—*Tube agglutination reaction of 35 aerobic, nonpathogenic neisseria in 16 prepared antiserums.*

Antigen		Antiserum															
		157	155	7925	2AMS	54	12	24	20–1	22	14	10–1	55	4	1485	4–2	9913
N. flavescens	157	2560	5120	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. flavescens	155	640	5120	—	—	—	—	—	—	—	200	—	—	—	—	—	—
N. flavescens	9746	640	5120	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. perflava	7925	—	320	2560	—	—	2560	200	640	400	—	400	320	—	—	—	—
N. perflava	2AMS	—	—	320	1600	2560	640	400	1280	640	800	—	—	—	—	—	—
N. perflava	54	—	—	—	400	2560	2000	3200	1280	640	—	—	—	—	—	—	—
N. perflava	12	—	—	800	400	1280	1000	320	400	200	400	640	800	—	—	—	—
N. perflava	24	—	—	—	1600	5120	3200	5120	2560	5120	2560	2560	—	1000	—	—	—
N. perflava	20–1	—	—	U	U	U	1000	U	1280	1280	U	U	U	U	—	—	—
N. perflava	22	—	—	—	2560	2560	U	2560	1280	1280	1280	U	U	1000	—	—	—
N. perflava	14	—	—	200	5120	5120	3200	5120	1280	2560	5120	U	320	U	—	—	—
N. perflava	10–1	—	—	—	2560	5120	800	2560	5120	2560	1600	2560	—	1000	—	—	—
N. perflava	1196	—	—	—	1000	5120	1000	3200	1000	1280	2560	3200	—	640	—	—	—
N. perflava	17	—	—	—	5120	5120	1000	5120	2560	2560	1600	5120	—	3200	—	—	—
N. perflava	28	—	—	—	—	320	—	—	—	—	320	—	—	—	—	—	—
N. perflava	8	—	—	—	—	800	—	—	—	—	—	—	—	—	—	—	—
N. perflava	31R	—	—	—	200	1000	200	U	U	U	U	U	U	U	U	U	U
N. perflava	0601	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
N. perflava	0765	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
N. flava	55	—	—	—	—	—	—	—	—	—	—	—	1280	—	—	—	—
N. flava	4	—	—	—	—	—	—	—	—	—	—	—	—	5120	—	—	—
N. flava	3–1	—	—	—	—	—	—	—	—	—	—	—	200	—	—	—	—
N. sicca	1485	—	—	—	—	—	—	—	—	—	—	—	—	—	2000	640	—
N. sicca	4–2	—	—	—	—	—	—	—	—	—	—	—	—	—	1280	2560	3200
N. sicca	9913	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
N. spp.	GP3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. spp.	GP16	—	—	320	—	—	—	—	—	—	—	—	—	—	—	—	—
N. spp.	F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N. catarrhalis	8176	All catarrhalis strains exhibited an instability in saline.															
	8193, 7900																
	0650, 01197																
	MF1, MF2																

U = Unstable suspension in saline.

TABLE 3.—*Absorption of* N. perflava *antiserums with* N. perflava *strains.*

Antiserum		Organism used for absorption	Agglutination titer with *N. perflava* strains									
Number	Treatment		2AMS	54	24	12	10–1	7925	1196	17	14	22
2 AMS	Unabsorbed		1600	400	1600	400	2560	—	1000	5120	5120	2560
	Absorbed	2AMS	—	—	—	—	—	—	—	—	U	U
		54	160	—	—	160	—	—	—	—	U	U
54	Unabsorbed		2560	2560	5120	1280	5120	—	5120	5120	5120	2560
	Absorbed	54	—	—	1280	160	—	—	1280	—	U	U
		24	—	640	—	—	—	—	—	—	U	U
		10-1	160	1280	2560	640	—	—	2560	1280	U	U
		1196	—	320	—	—	—	—	—	—	U	U
24	Unabsorbed		400	3200	5120	320	2560	200	3200	5120	5120	2560
	Absorbed	24	—	—	—	—	—	—	—	—	U	U
12	Unabsorbed		640	2000	3200	1000	800	2560	1000	1000	3200	U
	Absorbed	12	—	—	—	—	—	—	160	—	U	U
10-1	Unabsorbed		—	—	2560	640	2560	400	3200	5120	U	U
	Absorbed	10-1	—	—	—	640	1280	—	320	640	U	U
7925	Unabsorbed		320	—	—	800	—	2560	—	—	200	—
	Absorbed	7925	—	—	—	—	—	160	—	—	U	U

U = Unstable suspension in saline.

characterized as fermenting all or nearly all of the five carbohydrates and producing a pigment, constituted another serological group. Cross reactions among perflava strains were easily demonstrated, but the flava strains and other fermentative strains, i.e., *N. sicca*, demonstrated no agglutinin titers in perflava antiserums. The perflava strain 7925, which does not ferment maltose, demonstrated degrees of agglutination in some perflava antiserums (12, 24, 20-1, 22, and 10-1). However, the perflava strains demonstrated only slight agglutinability in the antiserum prepared against 7925. As previously reported by Elser and Huntoon (1909), it also was found that certain chromogenic strains, particularly 20-1 and 22, varied in their ability to form stable suspensions in saline. This observation is indicated in table 2. Strains 0601 and 0765 were continually rough, and strain 31R became rough during this study and remained so. The close antigenic relationship of some of the perflava strains, as determined by cross-absorption tests, is summarized in table 3.

Insufficient data were obtained to indicate a serological relationship among those pigmented strains which fermented some of the carbohydrates but which failed to ferment sucrose, namely *N. flava*. The cross agglutination which various strains of *N. perflava* exhibited in antiserums prepared against the flava strains 55 and 4 (table 2) is evidence of serological relationship of perflava strains to these flava strains. The flava strains were sufficiently different as to be serologically separated from the flavescens and perflava groups.

The two strains of *N. sicca*, 1485 and 4-2, which fermented all carbohydrates and gave no evidence of pigmentation, presented serological evidence for the possible establishment of another group. As shown in table 2, strains 1485 and 4-2 cross agglutinated; in addition, complete reciprocal absorption resulted between the two strains. The extreme variability of strain 9913, labeled *N. sicca*, in its fermentative capacity would appear to preclude any serological relationship with either the sicca group or the chromogenic groups.

All of the strains of *N. catarrhalis* were observed to spontaneously aggluti-

nate in the buffered saline and therefore could not be studied by the agglutination procedure described. Nevertheless, rabbits were injected with the *N. catarrhalis* strain 8193. This prepared antiserum failed to cross agglutinate with any of the other strains of neisseria tested.

SUMMARY AND CONCLUSIONS

The results presented indicate that some of the species of aerobic nonpathogenic neisseria as classified in *Bergey's Manual* (Breed et al, 1948) are serologically related. This does not imply that all strains of a given species which we have studied are serologically identical. Neither cross agglutination in an antiserum to the strength of its homologous strain nor complete reciprocal absorption was always achieved with strains considered to be equivalent in biochemical and serological characteristics.

This investigation has considered representative strains of each species, with the exception of *N. subflava*, a species which was not isolated at the University of Maryland and was not available in collections known to us. The serological differences together with biochemical and pigmentation characteristics suggest certain groups among the aerobic saprophytic neisseria. The nonchromogenic strains fall into two groups: (1) *N. catarrhalis* and (2) *N. sicca*. The chromogenic strains comprise at least three groups: (1) fermentative strains, (2) *N. flavescens*, and (3) guinea pig isolates of Pelczar et al (1949).

As previously indicated, aerobic saprophytic neisseria are primarily differentiated on a basis of chromogenesis and carbohydrate fermentation. However, the difficulties which arise in the interpretation of neisseria pigments and the variations in carbohydrate fermentation necessitates consideration of other characteristics in the classification of the neisseria. The results of this serological study offer an additional means of differentiation, which in general can be correlated with chromogenesis and carbohydrate fermentation.

The present observations have not altered the general agreement that *N. catarrhalis*, which is nonchromogenic and biochemically inactive by usual diagnostic procedures, is a valid species. Two strains, 1485 and 4-2 (*N. sicca*), were nonchromogenic, fermented the carbohydrates glucose, fructose, maltose, and sucrose, and were serologically related. Thus from the results obtained with these two strains, *N. sicca* would appear to be a distinct species.

The designation of a new species, *Neisseria caviae*, for the guinea pig isolates (Pelczar, 1951) is substantiated by their complete absence of serological relationship to the flavescens, perflava, or flava groups and by their inability to ferment carbohydrates. *N. caviae* and *N. flavescens* are easily distinguished from *N. catarrhalis* by their chromogenesis. Although differences in pigments of these nonfermentative species was previously indicated (Branham, 1951), the lack of serological relationship to *N. flavescens* was the more reliable means of differentiation of *N. caviae*.

In view of the serological data presented, the validity of three species (*N. perflava*, *N. flava*, and *N. subflava*) among the fermentative chromogenic strains of aerobic saprophytic *Neisseria* becomes questionable. Fermentative chromogenic strains which were sucrose-negative (*N. flava*) deviated sufficiently from the serological pattern of the perflava group as to be distinguished by agglutination tests. However, a serological relationship of perflava strains to flava strains has been indicated. It is suggested, therefore, that the name

Neisseria pharyngis be adopted for the fermentative chromogenic strains of aerobic nonpathogenic neisseria. This suggestion is in line with the opinion expressed by the British investigators. The perflava and flava groups described in this study could be assigned as varieties of *N. pharyngis*.

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